Behavior and Immunity in *Drosophila melanogaster*

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Abstract

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Immunity, behavior, and circadian regulation are important ways that animals maintain homeostasis. Defects in these physiologies often lead to disease or even death, yet many questions remain about how these physiologies are related. I explored the interactions between innate immunity, behavior, and circadian regulation by using *Drosophila melanogaster*, a convenient, genetically tractable model organism with both functionally and molecularly conserved innate immune and circadian clock systems. In the first chapter, I show that feeding, a circadian-regulated behavior, increases immunity to a sepsis-like infection. In the second, I present evidence suggesting that aging-related changes in immunity may be linked to circadian defects. Finally, I use a novel automated method to demonstrate that reduced grooming is a conserved sickness behavior in *Drosophila*.

The feeding project ultimately showed that mutating TORC2 components could increase the host's ability to kill and clear a bacterial infection, as well as survive the pathogenic effects of infection. Therefore we have identified a possible drug target to create host-based therapies for sepsis patients. We also have established *Drosophila* as a model system for studying a conserved sickness behavior: reduced grooming. This experimental paradigm will allow researchers to isolate mutants that do not show reduced grooming, and investigate whether this sickness behavior is adaptive or not.

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Finally, thank you to all of my labmates, who made my graduate school experience the enjoyable and enriching experience that it was.

Dedication

To my family. Thank you for the countless advantages you've provided me. I am so lucky to have known each of my wonderful and unique grandparents. I also thank my brother, mom, and dad for much love and support. This is for you.

I. Introduction

Infection by microbes is a constant threat to animals. The general question that guided my thesis research was how circadian-regulated physiologies contribute to survival of infection. Both innate immunity and the circadian clock are evolutionarily conserved between *Drosophila* and mammals, making the fruit fly an ideal model system for investigating how these two systems interact. My introduction is divided into five parts. In the first two, I'll introduce innate immunity and circadian regulation, topics that relate to the entire thesis. The next three sections will address the specific questions relevant to my research chapters.

Immunity in *Drosophila*

Immunity is the set of responses used by an organism to maintain homeostasis during infection. Immunity allows an organism to defend itself against pathogens, including viruses, bacteria, fungi, and parasites. There are two generally recognized types of immunity, innate and adaptive, with crosstalk existing between the two [4]. While vertebrates exhibit both types, invertebrates such as *Drosophila* lack an adaptive immune system. This makes the fruit fly a useful model to study innate immunity specifically. Innate immunity is the first line of defense when any animal is exposed to a new pathogen. This defense includes the barrier epithelia as well as responses that can be activated without prior exposure to a specific pathogen.

Innate immune mechanisms work in two ways to limit and prevent damage from invading microbes. Resistance mechanisms directly limit microbial growth, while tolerance mechanisms limit the pathogenic effects of microbial infection. It's easy to imagine how resistance mechanisms benefit host survival: they destroy the invading microorganisms. It is less intuitive how tolerance mechanisms benefit host survival without reducing pathogen load, but several possibilities exist. Some tolerance mechanisms may act to repair damage caused by the pathogen or the host's immune response, while other tolerance mechanisms may prevent said damage from occurring in the first place.

Although resistance has been deeply explored in the fly, less is known about tolerance. My work presented in Chapter II expands our knowledge of tolerance, demonstrating that circadian *Period* mutants

show increased tolerance of a specific bacterial infection, that dietary glucose and amino acids contribute to tolerance, and that the Target of Ramapmycin Complex 2 (TORC2) component Sin1 can inhibit tolerance. Research presented in Chapter III shows that aging reduces both tolerance and resistance in flies.

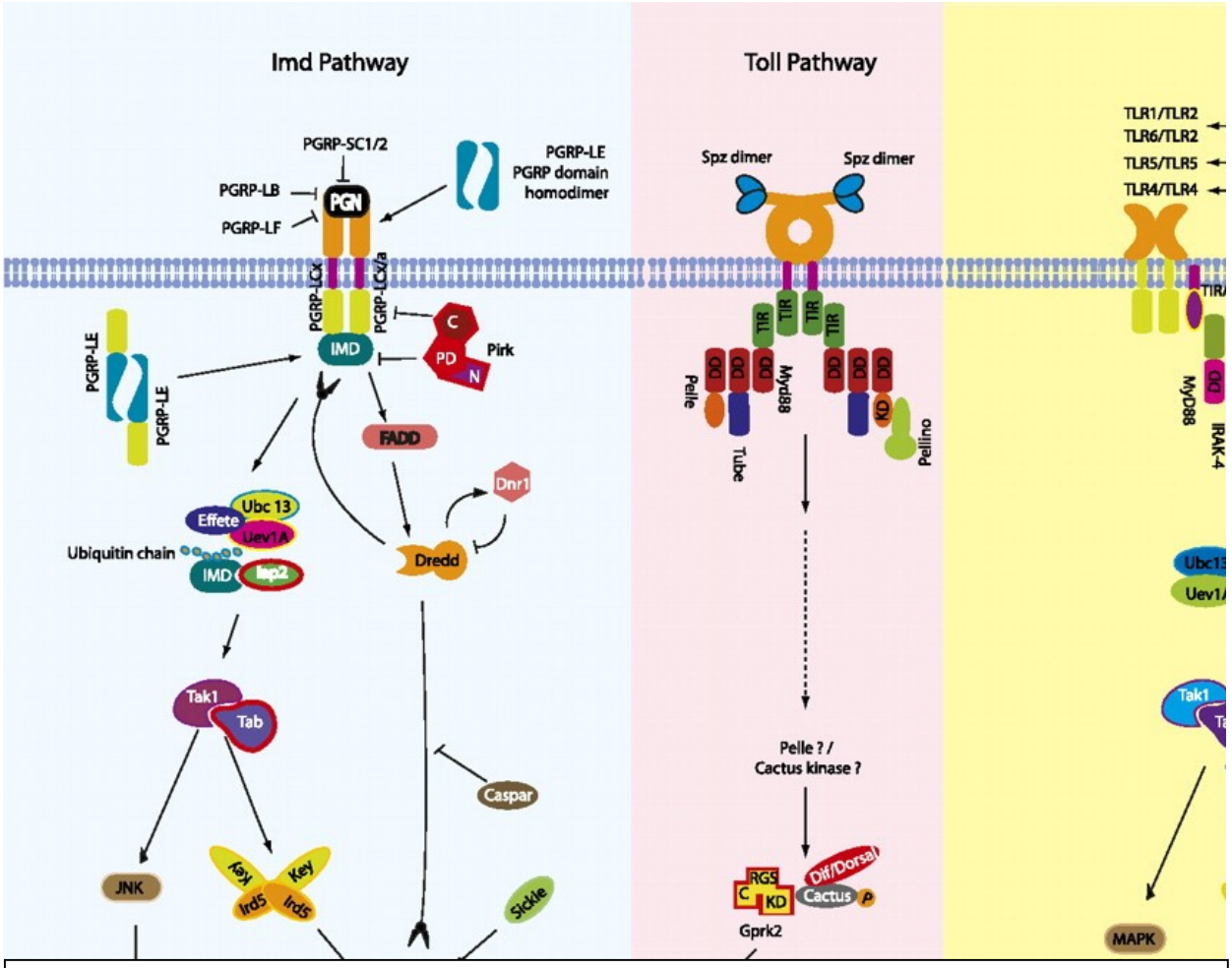

Figure 1. A high degree of homology exists between *Drosophila* and mammalian innate immunity. *Drosophila*'s Toll and Imd pathways and the mammalian TLR pathway. Similar shapes signify homology [1].

Resistance is mediated by *Drosophila*'s main immune signaling pathways: Toll and Imd, which share a high degree of homology with the mammalian TLR immune pathway (Figure 1). In fact, TLR stands for Toll-like receptor and alludes to the fact that the Toll receptor was first identified as a mediator of immunity in *Drosophila*. All three pathways are activated by the detection of pathogen-associated molecular patterns (PAMPs) and eventually result in the translocation of NFκB transcription factors into

the nucleus. Toll is thought to mostly mediate signaling in response to gram-positive bacteria and fungi, while Imd is thought to mediate the response to gram-negative bacteria [5]. In reality, there exists some crosstalk between the two pathways [6].

Three different resistance mechanisms have been characterized in *Drosophila*, which I will describe briefly here. Antimicrobial peptides (AMPs) are small proteins of 10 kDa or less which show antimicrobial activity [5, 7, 8]. Most AMPs are cationic and amphipathic, and thought to work by forming transmembrane pores in the microbes [9]. Different AMPs specifically target different types of pathogens, such as gram-positive bacteria, gram-negative bacteria, and fungi, by recognizing specific molecular structures on the surface of the microbe [9]. *Drosophila* produce at least seven kinds of AMPs [7], by induction in the fat body (orthologous to the mammalian liver) and constitutively in epithelial tissue. Like invertebrates, mammals also produce AMPs. In fact, over 100 AMPs have been discovered in humans [10] In *Drosophila*, induced transcription of AMPs is often used as a read out for activation of the Toll or Imd pathways.

In addition to AMP induction, *Drosophila* also activate an enzymatic cascade to cope with infection. This process, called melanization, results in the synthesis of melanin and reactive oxygen species (ROS) [11-15]. The melanin forms a capsule around the site of wounding or infection, which is thought to serve as a physical barrier against the spread of pathogens. The enzymes involved in melanin production also generate ROS as a by-product, which can cause damage to both host tissue and pathogens [14]. Although mammals do synthesize melanin, there is no evidence that this process represents an immune mechanism. But like *Drosophila*, mammals sometimes respond to wounding and infection by generating a burst of reactive oxygen species from hemocytes [16] .

The final resistance mechanism, examined in more detail in Chapter III, is phagocytosis. In this process, specialized blood cells called phagocytes recognize specific antigens on the surface of microbes, triggering engulfment of the microbe in a compartment called a phagosome, where they are destroyed [17]. Some *Drosophila* phagocytes circulate in the hemolymph, while others are sessile and clustered around the dorsal vessel/heart tube. Phagocytosis is functionally and, to some degree, molecularly conserved between fruit flies and mammals [18]. In mammals, white blood cells serve the same purpose as hemocytes in *Drosophila*. Previous experiments have shown that *Drosophila*

phagocytes are important for survival of infection by *Streptococcus pneumoniae* [19]. In Chapter III, I investigate phagocytosis in aged flies.

The research I conducted for my dissertation is separated into three chapters exploring different aspects of innate immunity. The first examines how circadian regulation, metabolism, and TOR signaling affect tolerance and survival of bacterial infection. The second chapter surveys how aging affects innate immunity and how these changes, collectively termed immunosenescence, might be related to the circadian changes caused by aging. Finally, the last chapter presents research on a form of sickness behavior during bacterial infection.

Circadian regulation in *Drosophila*

Circadian-regulated physiologies are implicated in immunity [20], but the precise molecular mechanisms that link the two are unknown. Like the first section on immunity, this section of the introduction will provide general background information on circadian regulation. This will be important for contextualizing all of the research in the thesis, and will be followed by three chapters outlining the specific questions addressed in my thesis research.

Circadian rhythm is the pattern of physiological changes that oscillate over ~24 hour periods and persist even under continual “free-run” conditions such as constant darkness. Internal circadian clocks are evolutionarily advantageous because they allow organisms to anticipate daily environmental changes in light, temperature, food, and mate availability. As a result, they are well-conserved in the animal kingdom. Circadian rhythms in both vertebrates and *Drosophila* are controlled by clock proteins, transcriptional regulators that themselves oscillate in activity and abundance over ~24 hour periods [3]. The central clock proteins in *Drosophila* are Clock (Clk), Cycle (Cyc), Period (Per), and Timeless (Tim). During the part of the day when Per and Tim are abundant, they form a heterodimer that translocates into the nucleus. There they inhibit their own transcription, preventing the heterodimer formed by Clk and Cyc from binding to promoter regions.

The stability of the Per/Tim heterodimer and its ability to enter the nucleus are dependent upon phosphorylation. Per and to a lesser extent Tim are phosphorylated by several different kinases, most notably Doubletime (Dbt), but also Casein Kinase 2 (CK2), and Shaggy (Sgg). They are de-

phosphorylated by at least two phosphatases, including Protein Phosphatase 2A (PP2A) and Protein Phosphatase 1 (PP1). Once Per is heavily phosphorylated, it interacts with an E3 ubiquitin ligase called Slimb which marks it for destruction by the proteasome.

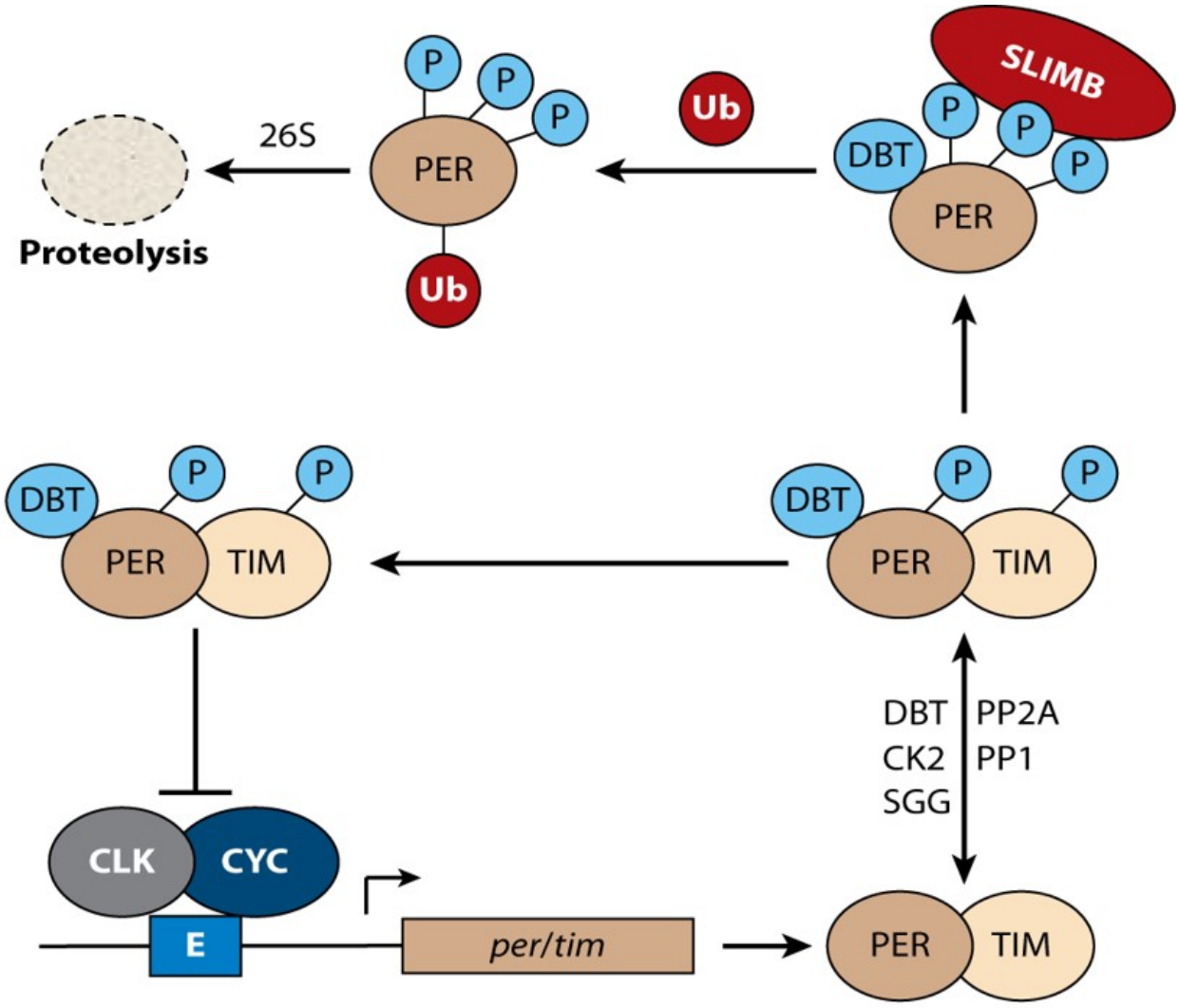

Figure 2. The core circadian clock of *Drosophila* contains an auto-regulatory negative feedback loop. The core molecular clock in *Drosophila*. CLOCK/CYCLE (CLK/CYC) bind to E-box elements (E) contained in the promoters of *period* (*per*) and *timeless* (*tim*). PER and TIM proteins are modified by the kinases DOUBLETIME (DBT), CASEIN KINASE 2 (CK2), and SHAGGY (SGG) and the phosphatases PROTEIN PHOSPHATASE 2A (PP2A) and PROTEIN PHOSPHATASE 1 (PP1). PER and TIM dimerize and transition to the nucleus, where they repress CLK/CYC activity. Phosphorylated PER and TIM also bind the E3 ubiquitin ligase SUPERNUMERARY LIMBS (SLIMB), which leads to ubiquitination and ultimately proteolysis by the 26S proteasome [3].

In addition to regulation by the negative feedback loop described, clock oscillations are entrained to cues from the animal's outside environment, called Zeitgebers. Light is one input known to be important for entrainment. When activated, the blue light receptor Cryptochrome (Cry) destabilizes Tim protein [21]. Additionally, certain phosphorylation sites on Per were recently shown to be light-sensitive [22]. Peripheral clocks in *Drosophila* are synchronized to a central clock, located in the large and small groups of the ventral cluster of lateral neurons (lLVn and sLVn). The central clock synchronizes the peripheral clocks by secreting a peptide called Pigment-Dispersing Factor (PDF) [23, 24].

The circadian machinery of *Drosophila* and mammals is largely comparable. In mammals, the circadian clock is located in the suprachiasmatic nucleus (SCN) in the hypothalamus [25]. All four main proteins of the negative feedback loop are conserved between *Drosophila* and mammals, though Cyc is called BMAL1, and in some cases, copy numbers differ [25]. Cry does not appear to be light sensitive in mammals, and plays the role of Tim in *Drosophila* [25]. The mammalian homolog of PDF is Vasoactive-Intestinal Polypeptide (VIP) [26].

In contrast to the cell-autonomous dynamics and regulation of the clock proteins themselves, much less is known about the molecular mechanisms underlying their downstream outputs, or circadian-regulated physiologies. These include locomotor activity [27], sleep [28], and feeding [29], as well as temperature preference [30], larval eclosion [31], and susceptibility to infection [20]. As previously stated, circadian rhythms are thought to be important because they allow animals to be in the correct physiological state when specific circadian environmental conditions are present. Therefore it is somewhat surprising that circadian rhythm persists over generations in the controlled laboratory setting, where food and mate availability do not cycle over the course of 24 hours. This suggests that circadian regulation of autonomous internal processes may be just as important as regulation of responses to external stimuli. However, many of these circadian-regulated autonomous internal processes have yet to be characterized.

The research in Chapter II focuses on how some of these less well-understood downstream outputs of the circadian clock relate to immunity. I use an arrhythmic Per mutant to show that circadian-regulated feeding behavior impacts the ability of flies to survive bacterial infection. I further establish that TORC1 activity is a circadian-regulated output and that it affects survival of infection.

Both circadian physiologies and immunity become mis-regulated in specific disease states such as aging [32, 33]. Therefore, we can use such disease states to investigate the interface between circadian regulation and immunity. The next section will provide context for the study of aging in *Drosophila* and the following will introduce the outstanding questions related to circadian regulation and immunity.

Aging

The population of the United States 65 and over is expected to almost double between 2012 and 2050 [34]. Industrialized nations will face many new challenges associated with aging-related disease such as increased demand for health care and long-term residential care services. It is estimated that in developed countries, health care spending per capita is three to five times higher for patients 65 and older [35]. Thus there is currently great interest in improving the health of aging individuals. Aging is clearly a risk factor for many diseases including bacterial infection [36]. However, the underlying mechanistic reasons for disease susceptibility are not well-defined in many cases. This section will briefly characterize aging, provide background on what is known, explore present theories of its causes, and introduce a major question in the field. The next section will present specific aspects of aging that were explored during my research.

The term “aging” is popularly used to denote individual or organismal senescence. In humans, organismal senescence is characterized by progressive inability to maintain homeostasis in one or more organ systems, leading to extreme states that eventually result in death. Age-related decline in effectiveness and integration of the stress response pathways is thought to be a major reason for loss of homeostatic ability.

Although there is no consensus on its exact cause, aging has been studied for decades. Studies have yielded many ways of extending lifespan in the laboratory. For example, it’s well established that dietary restriction can prolong life in many organisms. The first report of lifespan extension by caloric restriction was an experiment in rats, published in 1935 [37]. Lifespan extension by caloric restriction has now been established in many model organisms, ranging from unicellular yeast to worms, flies, rodents, and primates [38]. This highly conserved phenomenon led researchers to investigate whether down-

regulating nutrient-sensing pathways had the same effect. Indeed, manipulating pathways involving insulin or insulin-like signaling (IIS), target of rapamycin (TOR), AMP kinase, or sirtuins can all improve lifespan [39]. Similarly to caloric restriction, reducing the protein, amino acid, methionine, or tryptophan components in an animal's diet can also increase lifespan [40].

Extending lifespan is not the only goal of aging research, as extending healthspan would do more to improve human quality of life. Healthspan is the length of time that an animal is free from serious illnesses, rather than just alive. While lifespan represents a binary measurement: dead or alive, healthspan measures the organism's functional ability as it ages. From a biomedical perspective, aging progresses straightforwardly in humans: development of physiological dysfunction (impairment) leads to functional limitations (e.g., reduced mobility), increased risk of disease and disability, decreases in productivity, loss of independence, a reduction in quality of life and, ultimately, death. Improved healthspan would allow for a larger portion of an individual's life to be spent without physiological dysfunction (See Figure 1[41]). Therefore, although lifespan is technically easier to measure, aging research focused on improving healthspan will be extremely important if we want to improve quality of life for the world's population.

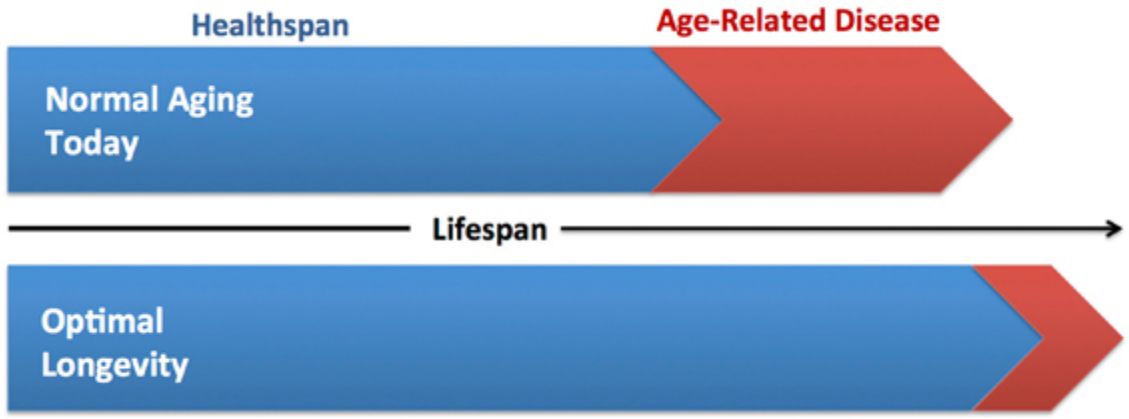

Figure 3. Increasing healthspan and optimal longevity. Comparison of current vs. ideal healthspan. Extending healthspan is a critical component of achieving optimal longevity, defined as living long, but with good health, function, productivity and independence [41].

Although a single cause of aging has not yet been identified, many promising theories exist. The cause of senescence on the cellular level has been extensively studied, and has yielded important insights into senescence in animals. It is known that cells in culture will undergo only a certain number of cell divisions. For example, in *Saccharomyces cerevisiae*, this limit is approximately 20 cell divisions [42]. This is called the replicative lifespan in yeast or the Hayflick limit in human tissue culture [43]. Experiments in cells have implicated genomic instability and dysfunctional mitochondria as major drivers of cellular aging [44, 45]. In fact, specific signaling pathways link DNA and mitochondrial damage [46], and these will be discussed in this section in more detail.

Currently, a major question in the field of aging is whether aging itself is an accidental process, caused by accumulated damage, or a programmed and purposeful process. Poetically, one author has proposed that aging is simply the result of the “imperfectness of each and every biological process…” [47]. Another theory is that since both cancer and longevity require robust populations of proliferating cells, the molecular processes that lead to aging were selected for because they tended to limit cancer [48]. Ultimately, both theories represent different interpretations of how natural selection shaped aging. Darwin posited that that each organism has limited energy available and that its resources must be concentrated in reproductive activity. Therefore, after reproductive capacity is exhausted, i.e., during aging, we can expect the first pathways to fail will be those that are energetically costly to maintain and lack redundancy.

DNA damage repair fits both of these criteria, and genomic instability is thought to be a major cause of cellular senescence. Damage can result from exogenous factors such as UV radiation, infrared radiation, and chemicals, or from endogenous factors, especially reactive oxygen species (ROS) generated during oxidative phosphorylation and replication errors [49, 50]. Specific DNA lesions which contribute to genomic instability include telomere shortening, thymine dimerization, interstrand crosslinks, spindle errors, double-stranded breaks, and base pair mismatch [50]. Genomic instability from DNA lesions can lead to the end of a cell’s replicative lifespan.

Mitochondrial damage and the pathways it triggers are also thought to be major drivers of cellular aging [51-54]. In addition to damaging DNA, ROS can damage proteins by causing undesired cross-linking and/or fragmentation [55]. Mitochondrial proteins and DNA may be especially susceptible to ROS

because mitochondria are the major producers of endogenous ROS, especially superoxide and hydrogen peroxide generated during oxidative phosphorylation [49].

Interestingly, although severe damage to mitochondria is detrimental, mild oxidative stress can have beneficial effects on mitochondria in many organisms [56]. This phenomenon is termed "mitohormesis". The word hormesis was coined to describe a Greek king who was convinced he would be poisoned. To avoid this fate, he drank small, increasing doses of poison thinking that this would protect him, should he fall prey to a poisoner. In more technical terms, hormesis means an adaptive response that exhibits a biphasic dose response [57]. With regard to mitochondria, mitohormesis is the theory that too much ROS is pathological, but a limited amount can be beneficial, because it causes the induction of protective pathways. Like the longevity-increasing interventions already discussed, mitohormesis is tightly linked to insulin signaling [56, 58]. In fact, the first single-gene mutation shown to extend lifespan was *daf-2* in *C. elegans* [59]. This protein was later shown to be involved in insulin-signaling [60] and extend lifespan through mitohormesis [61]. In addition to genetic manipulation of the insulin-signaling pathway, caloric restriction is also known to induce mitohormesis[62]. Given the essential metabolic processes (the Krebs/citric acid cycle and oxidative phosphorylation) that occur in mitochondria, it is perhaps predictable that insulin signaling should be tied to mitochondrial mechanisms of lifespan extension.

Less intuitively, recent work has shown that mitochondrial stress induces retrograde signaling from the mitochondria to the nucleus, altering the nucleus's transcriptional profile and putting the cell into a cytoprotective state [56]. Specifically, the mitochondrial unfolded protein response (UPR^{mt}) is thought to play a large role in lifespan-extension from mitohormesis [63]. Paradoxically, mitochondrial stress also causes an increase in the generation of ROS, which can act as signaling molecules to increase stress resistance, but can also be cytotoxic [64]. In short, complex bi-directional signaling between the nucleus and mitochondria has been extensively implicated in physiologies of aging [46, 53, 56].

While mitohormesis may happen to some degree in all cells, it seems to be especially relevant in tissues that are often implicated in age-related pathologies. For example, sarcopenia and cardiovascular disease are major age-related pathologies and manifest in the loss of skeletal muscle tissue and cardiac failure, respectively. Mitohormesis in muscle has been shown to promote longevity in *Drosophila* [58]. Mitohormesis has also been shown to increase longevity in cases of experimental cardiomyopathy in

mice [65]. This is perhaps unsurprising, given the crucial role of mitochondria in muscular metabolism. Mitochondrial metabolism is also implicated in cancer cells, which display an altered metabolic program known as the “Warburg effect” [66].

In humans, diseases of aging are often associated with increased inflammation. These include atherosclerosis, arthritis, cancer, diabetes, osteoporosis, dementia, vascular diseases, and metabolic syndrome [67]. Moreover, even in the absence of disease, a systemic, low-grade, chronic inflammation is observed with age [68]. Surprisingly, even though age-associated inflammation results from upregulation of specific immune effectors, infection is a major cause of mortality in the elderly. It has been estimated that over 900,000 seniors die of pneumonia every year in the U.S. [69]. Additionally, although seniors make up only one-fifth of the population, they make up two-thirds of the patients admitted to hospitals with sepsis [70]. Given the infection and inflammation phenotypes associated with aging, my thesis research focused specifically on how aspects of immune regulation change with age.

Aging in *Drosophila*

As previously mentioned, organismal senescence is characterized by loss of the ability to maintain homeostasis in one or more organ systems, which leads to disease states that eventually result in death. The innate immune system and circadian regulation are two systems which exhibit altered function with age. These systems are highly relevant to susceptibility to bacterial infection, which makes their senescence an important subject of study.

Since the pathology of aging is systemic as well as tissue-specific, experiments in whole organisms can yield unique insights. Unfortunately, simultaneously maintaining animal cohorts at varying ages is complicated and costly. This makes animals with a short lifespan well-suited to aging research. Standard wild-type *Drosophila* live no more than four months [71], while commonly used mouse strains have mean lifespans varying from 16 to 30 months [72]. In terms of conservation, dietary restriction is a well-known lifespan-extending environmental intervention in both mammals and *Drosophila*. Importantly, many pathways involved in mammalian aging are conserved in *Drosophila*, including insulin signaling [73], ROS-induced stress [74], and the TOR pathway [75]. Mutant strains for genes in these pathways have already been constructed and characterized.

Aging affects the innate immune system in complex ways [76-78]. Many aging-associated diseases, including Alzheimer's disease, atherosclerosis, diabetes, sarcopenia, and cancer are associated with increased inflammation [79]. The older population is highly susceptible to infections; one-third of deaths in people aged 65 and over are due to infectious disease [80]. But examining aging-associated changes in the innate immune system of vertebrates is complicated and has yielded conflicting results [33, 81]. Studying a simpler model system could help define the effects of aging on the innate immune system.

The effect of age on survival of bacterial infection has been studied to a limited extent in *Drosophila* [82, 83], but it remains to be determined whether survival of some or all pathogenic infections is reduced in old flies. Another important question is whether old flies have altered resistance or altered tolerance against those pathogenic bacteria. Studies investigating how age affects bacterial load after infection [82-84] have given inconsistent results. In Chapter III, I describe how old flies survive infection by different bacterial pathogens. I also show that aging can reduce both tolerance and resistance to bacterial infection.

Aging in humans is also associated with complex changes in circadian regulation. The two main aging-associated changes in circadian rhythm consistently reported in the literature are reduced consolidation in sleeping and wakeful periods [85] and a phase advance in the circadian cycle [86, 87]. In other words, the elderly are more likely to nap during the day and more likely to suffer from insomnia during the night. They also tend to wake up earlier in the morning and go to bed earlier at night. Aging-associated changes in circadian rhythm are clinically important because over half of elderly people report chronic sleep complaints [88]. Additionally, several age-related pathologies including Alzheimer's disease, Parkinson's disease, and stroke are associated with sleep disturbances [89]. Both fragmentation of rest/activity cycles and phase advance have been observed in mammalian models of aging [90, 91]. However, the mechanisms that underlie age-related sleep/activity fragmentation and phase advance are unclear. Moreover, the impact of these aging-associated changes in circadian regulation on immune system function and susceptibility to disease remains unknown.

In Chapter III, I explore how aging-associated changes in circadian regulation may be related to aging-associated changes in immunity. I also examine phagocytosis, a circadian-regulated immune mechanism already known to be altered with age in *Drosophila* [92, 93].

Nutrient signaling in *Drosophila*

Chapter II addresses several questions about how nutrient signaling affects immunity in flies. This section will provide background information to help the reader understand how this research expands knowledge in that field.

Anorexia is one infection-associated behavior conserved between mammals and *Drosophila*. But it's unclear whether reduced nutrient intake is generally helpful or harmful to bacteria-infected flies, since dietary restriction improves survival of infection by *Salmonella typhimurium* but reduces survival of *Listeria monocytogenes* infection [94]. In Chapter II, I establish that dietary restriction decreases survival of infection by *Burkholderia cepacia*, a clinically relevant pathogen. I also show that a circadian mutant with increased feeding behavior survives *B. cepacia* infection better, unless on a restricted diet.

Dietary intake includes both macronutrients, such as proteins and carbohydrates, as well as micronutrients, such as vitamins and minerals. While much is known about the role of micronutrients in human disease, less is known about the role of macronutrients. For example, the role of vitamin A, iron, and zinc in immunity have been well-established [95]. Controlled studies on the role of macronutrients are less straight-forward than dietary supplement versus placebo. For example, with circulating sugars, there continues to be debate about how aggressively to manage blood glucose in sepsis patients [96]. This is despite mammalian studies indicating that insulin application can be ineffective [97] or even harmful [98]. In *Drosophila*, insulin-like signaling (ILS) becomes dysregulated during the course of *Mycobacterium marinum* infection, but it's currently unknown whether ILS at the time of infection can affect the eventual outcome. Chapter II of my dissertation addresses the acute role of circulating sugar in survival of infection, showing that dietary glucose provided for a short time or injected glucose administered during a narrow window of time near infection can improve survival of bacterial infection. Experiments in Chapter II also show that dietary amino acids can confer a survival benefit during bacterial infection in *Drosophila*.

Like ILS, the TOR pathway senses nutrient availability and is important for maintaining energy homeostasis. In fact, the TOR pathway interacts with ILS, as seen in Figure 4. TOR stands for Target Of Rapamycin, rapamycin being a potent immunosuppressant used in human organ transplants. In vertebrates and *Drosophila*, the TOR kinase forms two distinct multimeric complexes, termed TORC1 and TORC2 [99].

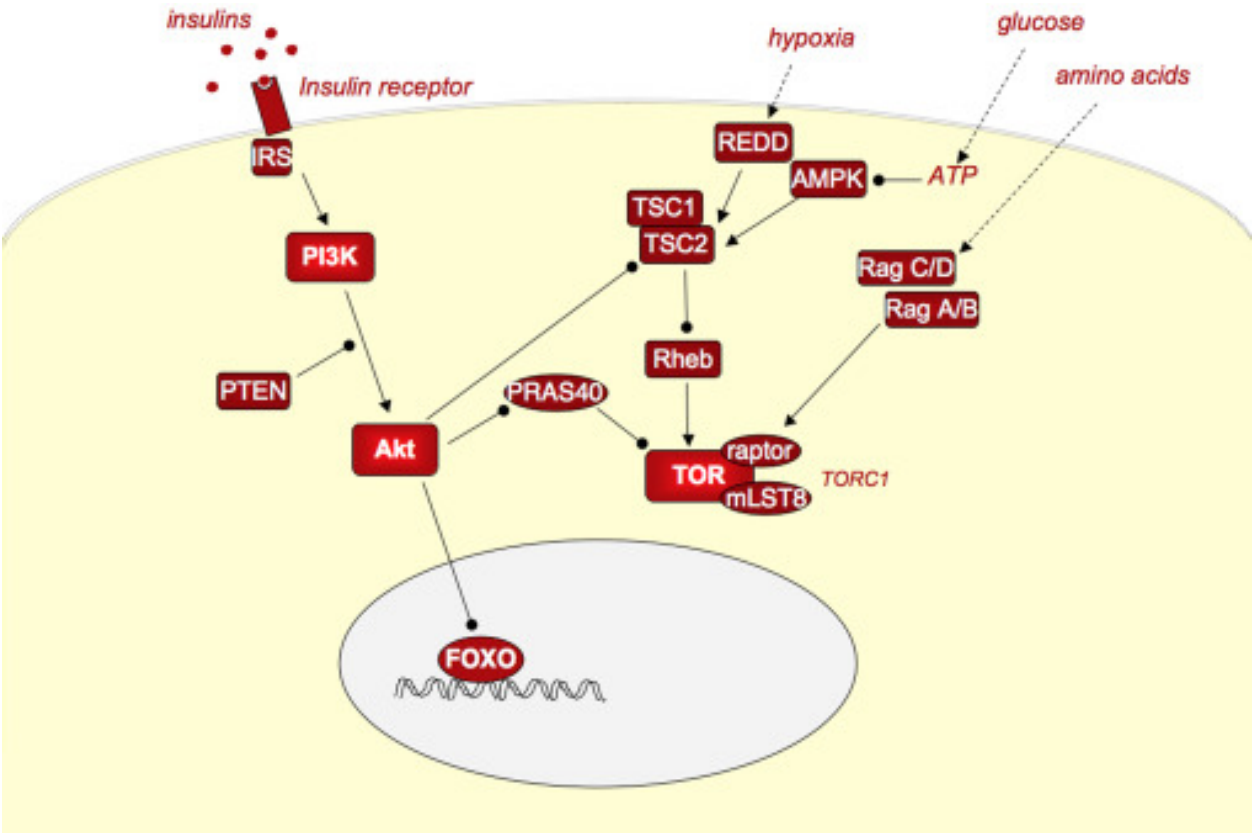

Figure 4. A schematic outline of the insulin/TOR pathways, showing those components conserved between Drosophila and mammals. The insulin/PI3K/Akt branch can regulate transcription through the regulation of the FOXO transcription factor. Central to the TOR branch is TORC1, a protein complex consisting of TOR kinase, raptor and mLST8. TORC1 is activated in response to amino acid, glucose and oxygen availability. TORC1 is also regulated by PI3K/Akt signaling. It's unclear how TORC2 signaling interacts with these pathways; TORC2 not shown. Arrows and bars indicate positive and negative regulation, respectively [2].

Both of the TOR complexes include the TOR kinase and Lst8, although Lst8 is thought to regulate in cell growth only when in TORC2 [100]. However, each complex also has distinct components, such as RapTOR in TORC1 [101, 102], or RicTOR and Sin1 in TORC2 [103, 104]. TORC1 signaling is stimulated by amino acids through Rac GTPases [105] and by growth factors via the PI3K pathway [106]. TORC1 regulates cell growth and translation through S6K and 4E-BP [102, 107]. The inputs of TORC2 signaling are not currently known, but the outputs are thought to include cytoskeletal rearrangement [108].

Despite the fact that TOR stands for “Target of Rapamycin”, only TORC1 is sensitive to rapamycin [108]. Rapamycin works as an immunosuppressant in vertebrates by inhibiting T-cells [109]. Since insects lack an adaptive immune system, rapamycin has not been extensively used in studies of insect immunity. Surprisingly, our work detailed in Chapter II shows that rapamycin has a similar immunosuppressive effect on flies and that TORC1 signaling is important for resistance to *B. cepacia* infection.

In contrast to TORC1, comparatively less is known about the effects of TORC2 signaling in general and in relation to immunity. In the absence of a pharmacological inhibitor specific to this branch of the TOR pathway, genetic manipulation is the preferred way to study TORC2. Conservation of TORC proteins makes the genetically tractable *Drosophila* system ideal. In Chapter II, I investigate the role of TORC2 signaling in immunity. I show TORC2 signaling inhibits both tolerance and resistance to bacterial infection.

Sickness Behavior in *Drosophila*

Sickness behavior is a coordinated set of changes in animal behavior that develops in individuals with infections. The work in Chapter IV of this thesis addresses two outstanding questions related to a specific sickness behavior, reduced grooming, in *Drosophila*. I show that reduced grooming behavior is a conserved sickness behavior in *Drosophila* and establish a system to study whether sickness behavior confers a benefit to the infected animal.

In mammals, sickness behavior manifests as a combination of depression/lethargy, anorexia, and reduced grooming behavior [110]. This combination of symptoms is seen during infections by bacteria,

viruses, and protozoa [110], and can also be observed during chronic inflammatory diseases. In vertebrates, sickness behavior is induced by pro-inflammatory cytokines and inhibited by glucocorticoids and calorie restriction [111-114].

Although both invertebrates and vertebrates exhibit sickness behavior, sickness behavior is best understood in farm animals where weight loss is likely to lead to revenue loss [115, 116]. A major question about sickness behavior is to what degree it is conserved in invertebrates, which only have innate immune systems. There is some evidence that insects, including *Drosophila*, display sickness behaviors. Although insects can't regulate their body temperature in the same way as vertebrates, behavioral fevers have been observed in crickets [117, 118], locusts [119], and bees [120]. There is also evidence of illness-induced anorexia in insects including caterpillars [121] and *Drosophila* [122, 123]. It's currently unknown whether reduced grooming is a conserved sickness behavior in insects.

Another major question in the field of sickness behavior is whether or not it serves an adaptive purpose. The ubiquity of sickness behavior in the animal kingdom suggests that it may confer a benefit. But sickness behaviors could instead be a result of debilitation, serving no adaptive purpose. In regard to whether sickness behaviors are adaptive, depression is well-studied [124]. Scientists have developed theories that depression could benefit a sick animal by limiting the spread of infectious agents, facilitating conservation of energy, and limiting the possibility of exposure to additional pathogens. Reduced grooming could also benefit a sick animal by facilitating conservation of energy. The resulting disheveled appearance could serve as a warning to one's community, preventing the spread of infectious agents. Unfortunately, most sickness behaviors are inextricable from fever in mammals. This makes it difficult to execute controlled studies on whether sickness behaviors benefit the host.

Grooming is any behavior related to body surface care and is widespread throughout the animal kingdom [125]. That grooming is universal despite opportunity cost to the animal indicates that grooming must be an essential part of animal physiology. Grooming has been studied for several decades in terrestrial vertebrates where its suggested functions include thermoregulation and communication [126-130].

Like terrestrial vertebrates, insects also devote resources to grooming [131]. However, the purpose of grooming in insects is still unclear. Removing of cuticular hydrocarbons or introduced dirt from

the body surface and distributing pheromones are some of the proposed functions [132-135]. Grooming may also function to prevent disease in insects, as immune-stimulating substances like LPS and dead bacteria are known to elicit grooming behavior in *Drosophila* on contact with the cuticle [136]. It's also been suggested that grooming functions in insects to maintain sensory organ acuity, or maintain adhesion on the attachment pads of the tarsi (feet) [137].

Grooming behavior occurs spontaneously, but can be altered through stimuli, including dust particles, chemical irritants, and weak mechanical stimulation [137, 138]. This raises the question of how this behavior is regulated. Recent work has identified specific neural circuits that can induce grooming upon mechanosensation and clarified that different neuronal layers elicit grooming for different durations [139]. Additionally, research in *Drosophila* has shown that removal of the D1 family dopamine receptor causes reduced grooming behavior [140], and overexpression of a zinc-metallopeptidase implicated in the control of neuropeptide levels causes increased grooming behavior [141]. Therefore, both specific circuits and neurotransmitters regulate grooming. Studying how grooming is controlled may provide insight into how genetic and neuronal systems regulate animal behavior, and furthermore, into how animal behaviors developed throughout evolution.

In Chapter IV of this thesis, I present evidence that *Drosophila* exhibit sickness-associated changes in grooming behavior, based on an automated video method developed by collaborators at the University of Miami. This is the first evidence that infected flies show reduced grooming behavior when infected with a bacterial pathogen, as mammals do. This method will be helpful for research into whether reduced grooming is an adaptive sickness behavior.

II. *Period*-regulated feeding behavior and TOR signaling modulate survival of infection [142]

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Abstract

Most metazoans undergo dynamic, circadian-regulated changes in behavior and physiology. Currently it is unknown how circadian-regulated behavior impacts immunity against infection. Two broad categories of defense against bacterial infection are resistance, control of microbial growth, and tolerance, control of the pathogenic effects of infection. Our study of behaviorally arrhythmic *Drosophila* circadian *Period* mutants identified a novel link between nutrient intake and tolerance of infection with *B. cepacia*, a bacterial pathogen of rising importance in hospital-acquired infections. We found that infection tolerance in wild-type animals is stimulated by acute exposure to dietary glucose and amino acids. Glucose-stimulated tolerance was induced by feeding or direct injection; injections revealed a narrow window for glucose-stimulated tolerance. In contrast, amino acids stimulated tolerance only when ingested. We investigated the role of a known amino acid-sensing pathway, the TOR (Target of Rapamycin) pathway, in immunity. TORC1 is circadian-regulated and inhibition of TORC1 decreased resistance, as in vertebrates. Surprisingly, inhibition of the less well-characterized TOR complex 2 (TORC2) dramatically increased survival, through both resistance and tolerance mechanisms. This work suggests that dietary intake on the day of infection by *B. cepacia* can make a significant difference in long-term survival. We further demonstrate that TOR signaling mediates both resistance and tolerance of infection and identify TORC2 as a novel potential therapeutic target for increasing survival of infection.

Introduction

Evolutionarily conserved circadian mechanisms regulate daily, dynamic changes in animal behavior and physiology [143]. The core circadian clock is composed of four transcriptional regulators paired as two heterodimers in an auto-regulatory transcriptional negative feedback loop (Hardin [144]. In *Drosophila*, Clock and Cycle form one heterodimer and Timeless (Tim) and Period (Per) form the other. Clock and Cycle are transcriptional activators, promoting the expression of *Tim* and *Per* as well as hundreds of tissue-specific target genes [3, 143, 145]. Circadian oscillations in gene expression are thought to cause circadian oscillations in physiological function and ultimately organismal behavior.

We previously found that *Drosophila* innate immunity against *S. pneumoniae* infection is circadian-regulated [20] [146]. For both flies and vertebrates, innate immunity is the first line of defense against infection. *Drosophila* lack adaptive immune components such as T cells and B cells and rely on innate immune responses to survive infection [147]. Evolutionary conservation extends to the two primary *Drosophila* immune signaling pathways, the Toll and Imd pathways [148]. Flies and vertebrates employ several similar innate immune mechanisms to kill bacteria, including phagocytosis by immune cells, reactive oxygen species generation (melanization in flies), and secretion of antimicrobial peptides (AMPs).

Resistance is only one type of defense against bacterial infection. Resistance mechanisms such as the immune functions listed above control bacterial proliferation, reducing pathogenesis by decreasing the host's pathogen burden. A second distinct, complementary type of defense is termed tolerance [149, 150]. Tolerance physiologies allow the organism to survive the pathological effects of infection—caused by microbes or the host immune response—without necessarily decreasing bacterial load [151] [152].

Tolerance physiologies are not well understood, but include feeding and metabolism. In *Drosophila*, decreased survival of infection for two bacterial pathogens, *M. marinum* or *L. monocytogenes*, is associated with decreased metabolic stores [153, 154]. The effect of feeding behavior on infection is pathogen-specific: decreased feeding increases survival of *S. typhimurium*, *E. coli*, and *E. caratova* infections, but decreases survival of *L. monocytogenes* infection [94, 155]. In most cases, the precise nutrients important for survival and underlying molecular signaling pathways have not been identified.

Both feeding behavior and metabolic gene expression are circadian-regulated, and both fly and mouse circadian mutants exhibit metabolic disorders and altered feeding behavior [156, 157]. While we and others have shown previously that host resistance against specific pathogens is circadian-regulated, it is not clear whether loss of circadian-regulated metabolism and feeding behavior affect immunity against infection [20, 146, 158].

Here we exploit a rapid, lethal infection of *Drosophila* with the human pathogen *Burkholderia cepacia* to examine how acute differences in feeding behavior and diet impact infection tolerance. *B. cepacia* is a significant cause of hospital-acquired infection and tolerance mechanisms increasing survival of this infection are currently unknown [159]. We found that Per^{01} circadian mutants have increased tolerance to infection with *B. cepacia* and that increased tolerance is dependent on increased nutrient intake. In wild-type flies, infection tolerance is stimulated by influx of dietary glucose and amino acids at the time of infection. Because the TOR pathway is a classic amino-acid sensor, we asked whether TOR kinase mediates infection tolerance [160]. TOR associates with two related but distinct complexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2), which in some contexts have opposite effects [161, 162]. We found that TORC1 activity is circadian-regulated and that TORC1 activates resistance, as observed in vertebrates [109]. In contrast, the less well-characterized TORC2 had the opposite effect on survival and inhibits both resistance and tolerance. This work suggests that specific pharmacological TORC2 inhibitors could provide novel host-directed therapeutics for survival of infection.

Results

Period (Per^{01}) mutants are more tolerant of *B. cepacia* infection than wild type.

We found that arrhythmic Per^{01} *Drosophila* mutants survived longer than isogenic wild-type controls when infected with the human pathogen *Burkholderia cepacia*, a previously described infection model [163-165] (Figure 1A-B, $p<0.0001$). To determine whether this increased survival was due to altered resistance or tolerance, we measured bacterial loads of individual flies during infection. Whether the kinetics of survival were slow (over days, 18ºC) or fast (over hours, 29ºC), wild type and Per^{01} mutants carried equivalent bacterial loads (Figure1C-D, $p>0.05$ for each time point). This result suggests that the enhanced survival of Per^{01} mutants is not due to greater resistance, but due to greater host tolerance.

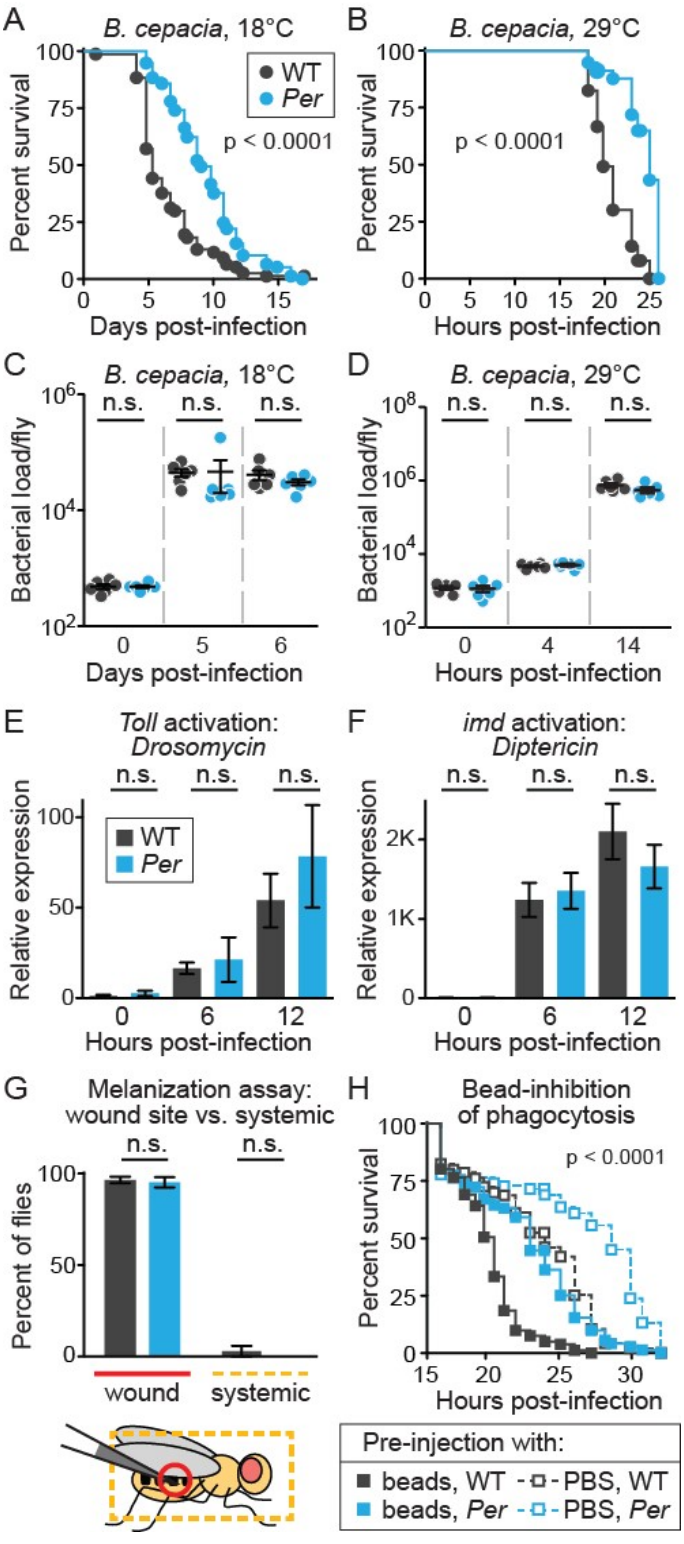

Figure 1: *Period* mutants exhibit greater tolerance than isogenic controls during infection with *B. cepacia*. Per^{01} mutants (blue) survived longer than wild type (dark grey) during A) a long infection (low dose at low temperature, 18°C; Per^{01}, n=78; WT, n=77, p<0.0001) and B) a short infection (high dose at high temperature, 29°C; Per^{01}, n=57; WT, n=64, p<0.0001) with *B. cepacia*. Per^{01} mutants and wild-type flies had similar bacterial loads over time following a C) long infection (n≥4 flies/time point, all n.s.) and D) short infection (n=6 flies/time point, all n.s.) with *B. cepacia*. Consistent with a tolerance phenotype, antimicrobial peptide (AMP) induction via the *Toll* and *imd* pathways did not differ between Per^{01} mutants and wild-type flies after *B. cepacia* infection as shown by: E) *Drosomycin* and F) *Diptericin* (n=3 samples of 6 flies each, all n.s.). Other AMPs are shown in Figure S1 (n=3 samples of 6 flies each, all n.s.). G) Per^{01} mutants and wild-type flies did not exhibit differences in systemic and injection wound site melanization after *B. cepacia* infection (3 trials, n=17-22 flies/trial/genotype, all n.s.). H) Inhibition of phagocytosis by bead pre-injection did not block the Per^{01} mutant survival advantage over wild type after *B. cepacia* infection (Per^{01}, n=76 with beads, n=81 with buffer; wild type, n=81 with beads, n=80 with buffer; p<0.0001 for all pair-wise curve comparisons except WT buffer vs. Per^{01} with beads, n.s.). p-values for survival curve comparisons were obtained by log-rank analysis; p-values for bacterial load comparisons were obtained using unpaired t-tests for 0 hour time points, while subsequent time points were tested with non-parametric Mann-Whitney U tests; p-values for AMP and melanization comparisons were obtained using unpaired t-tests; error bars represent the mean ± S.E.M.; n.s.=not significant (p>0.05).

Known resistance mechanisms do not explain increased survival of infection.

To confirm that *Per*^{01} mutants are more tolerant of *B. cepacia* infection, we analyzed three well-characterized resistance mechanisms following infection: antimicrobial peptide (AMP) induction, melanization, and phagocytosis. We found no significant differences between wild type and *Per*^{01} mutants in *B. cepacia*-induced AMP expression (Figure 1E-F, Figure S1A-E) or systemic melanization, typically not induced by *B. cepacia* (Figure 1G) [166]. While inhibition of phagocytosis by bead pre-injection decreased survival of both *Per*^{01} and wild-type controls (both $p<0.0001$), *Per*^{01} mutants still survived significantly longer than wild type (Figure 1H, $p<0.0001$), suggesting that phagocytosis is not responsible for the increased survival of *Per*^{01} mutants. Taken together, these results suggest that *Per*^{01} mutants have increased tolerance, not resistance, during *B. cepacia* infection.

***Per* mutants have decreased energy storage.**

We hypothesized that increased metabolic stores underlie the increased tolerance of *Per*^{01} mutants. Metabolic gene expression is circadian-regulated [156, 157], and increased metabolic stores underlie increased survival during infection with two other facultative intracellular bacterial pathogens, *M. marinum* and *L. monocytogenes* [153, 154]. If *Per*^{01} mutants have increased metabolic reserves, they should be less susceptible to starvation. In contrast, we found that *Per*^{01} mutants starve more quickly than wild-type controls (Figure 2A, $p<0.0001$), suggesting that *Per*^{01} mutants have fewer metabolic reserves than wild type. To test this, we measured three major types of energy storage: fat (triglycerides), glycogen, and circulating sugars (trehalose and glucose). Consistent with sensitivity to starvation, uninfected *Per*^{01} mutants had significantly lower levels of triglycerides ($p=0.0004$) and glycogen ($p=0.0007$), while trehalose and glucose levels were similar to wild type ($p=0.7065$) (Figure 2B).

Although *Per*^{01} mutants have lower metabolic reserves than wild type before infection, *Per*^{01} mutants may have higher metabolic reserves during infection. To test this, we measured metabolic reserves during *B. cepacia* infection. Both *Per*^{01} mutants and wild type lost energy stores during infection, but *Per*^{01} mutants maintained the same or lower energy stores than wild type (Figure 2C). At 16 hours post-infection, just before flies begin to die, triglyceride levels in *Per*^{01} mutants were still lower than wild type (~70% of wild type, $p=0.0001$), with levels of circulating sugars and glycogen similar to wild type

(p=0.9314 and 0.4804, respectively). These data indicate that the increased tolerance of *Per*^{01} mutants is not due to greater energy stores up until the lethal phase of infection.

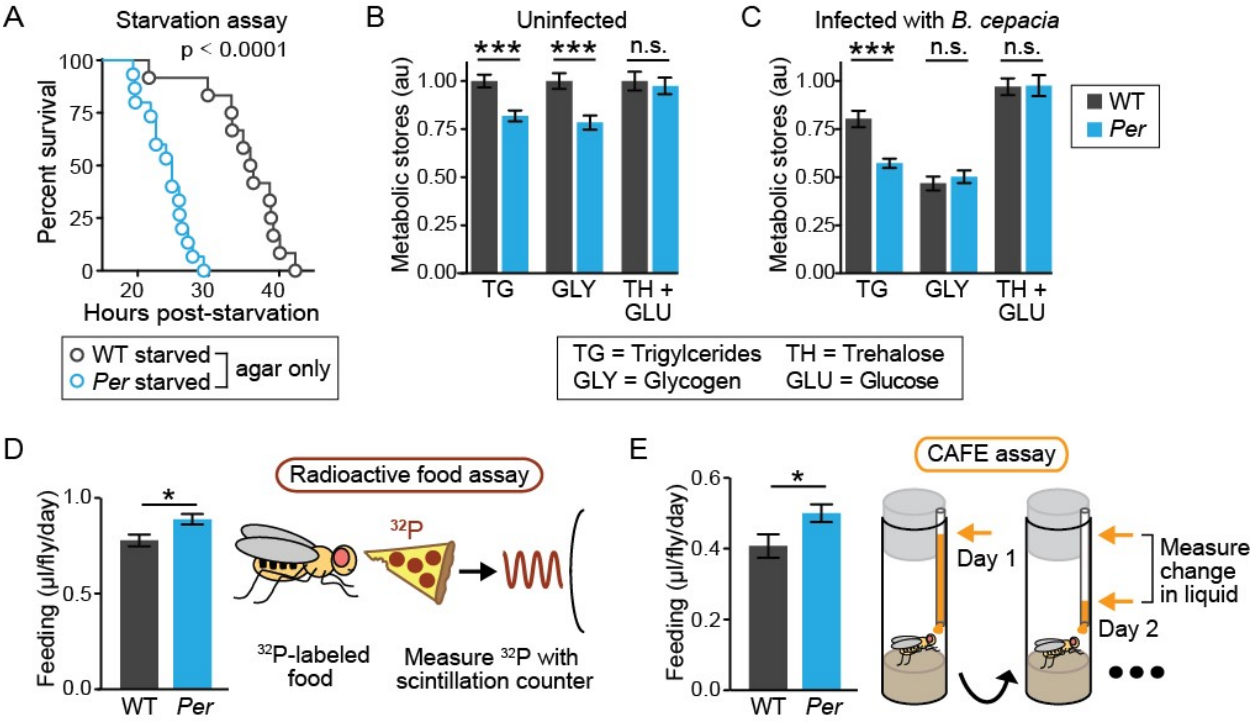

Figure 2: *Per*^{01} mutants have lower metabolic resources and eat more than wild-type flies. A) Uninfected *Per*^{01} mutants were more sensitive to starvation than uninfected wild-type flies (*Per*^{01} n=15; WT n=12; p<0.0001). B) Quantification of metabolic storage levels comparing uninfected *Per*^{01} mutants and wild-type flies (n=12 for both) revealed that *Per*^{01} mutants had lower levels of triglycerides (p=0.0004) and glycogen (p=0.0007) and similar levels of primary circulating sugars (n.s.). C) 16 hours after infection with *B. cepacia*, *Per*^{01} mutants relative to wild type (n=12 for both) had lower levels of triglycerides (p=0.0001) and similar levels of glycogen and primary circulating sugars (both n.s.). D) In the radioactive food assay, *Per*^{01} mutants ate ~14% more than wild type (*Per*^{01} n=9; WT, n=9, p=0.016). E) In the CApillary FEeder (CAFE) assay, *Per*^{01} mutants ate ~23% more than wild type (*Per*^{01}, n=24; WT, n=21; p=0.034). p-values were obtained by unpaired t-test; error bars represent the mean ± S.E.M.; n.s.=not significant (p>0.05); *=p≤0.05; ***=p≤0.001.

***Per*^{01} mutants exhibit increased feeding behavior.**

Because *Per*^{01} mutants have low metabolic reserves, we hypothesized that they eat more than wild type and that this increased feeding itself enhances infection tolerance. To test this, we measured the consumption of ^{32}P-labeled, solid food (Figure 2D)[167, 168] and liquid food using the Capillary Feeder (CAFE) assay (Figure 2E) [167, 169]. In the ^{32}P-labeled food assay, *Per*^{01} mutants ate 14% more than wild type; in the CAFE assay, *Per*^{01} mutants ate 23% more than wild type (Figure 2D, p=0.016; Figure 2E, p=0.034). These results resemble those of Xu et al. with flies expressing a dominant-negative form of

Clock (another core circadian regulator) in metabolic tissues [156]. Thus *Per*^{01} mutants exhibit significantly greater food intake than wild type.

Nutrient availability enhances infection tolerance of *Per*^{01} mutants.

If the increased survival of *Per*^{01} mutants is due to increased feeding, then decreasing nutrient intake by dietary restriction should abolish the enhanced survival time of *Per*^{01} mutants after *B. cepacia* infection. To restrict dietary intake, flies were fed a low sugar, protein-free diet containing only water, agar, and 1% glucose ~24 hours before and during infection and compared to flies on standard diet (Figure 3A). We found that this restricted diet decreased survival time after high-dose infection for both wild type (Figure 3B, $p<0.0001$) and *Per*^{01} mutants (Figure 3D, $p<0.0001$). *Per*^{01} mutants survived significantly longer than wild-type flies when fed standard food (20/20 experiments), with an average of 22% increased median survival time. In contrast, diet-restricted *Per*^{01} mutants either had no survival advantage over wild type (4/12 experiments), survived significantly less well than wild type (2/12 experiments), or survived an average of only 7% longer than wild type (6/12 experiments) (Figure 3F). Bacterial loads remained unchanged under all feeding conditions (Figure 3C,E,G; $p>0.05$ for all time points). Thus dietary restriction decreases host tolerance of infection. While we cannot exclude the possibility that dietary restriction overrides differences between *Per*^{01} mutants and wild type by a different mechanism than that causing increased tolerance in *Per*^{01} mutants, these results suggest that the increased feeding behavior of *Per*^{01} mutants on the day of infection contributes to their increased tolerance of *B. cepacia* infection.

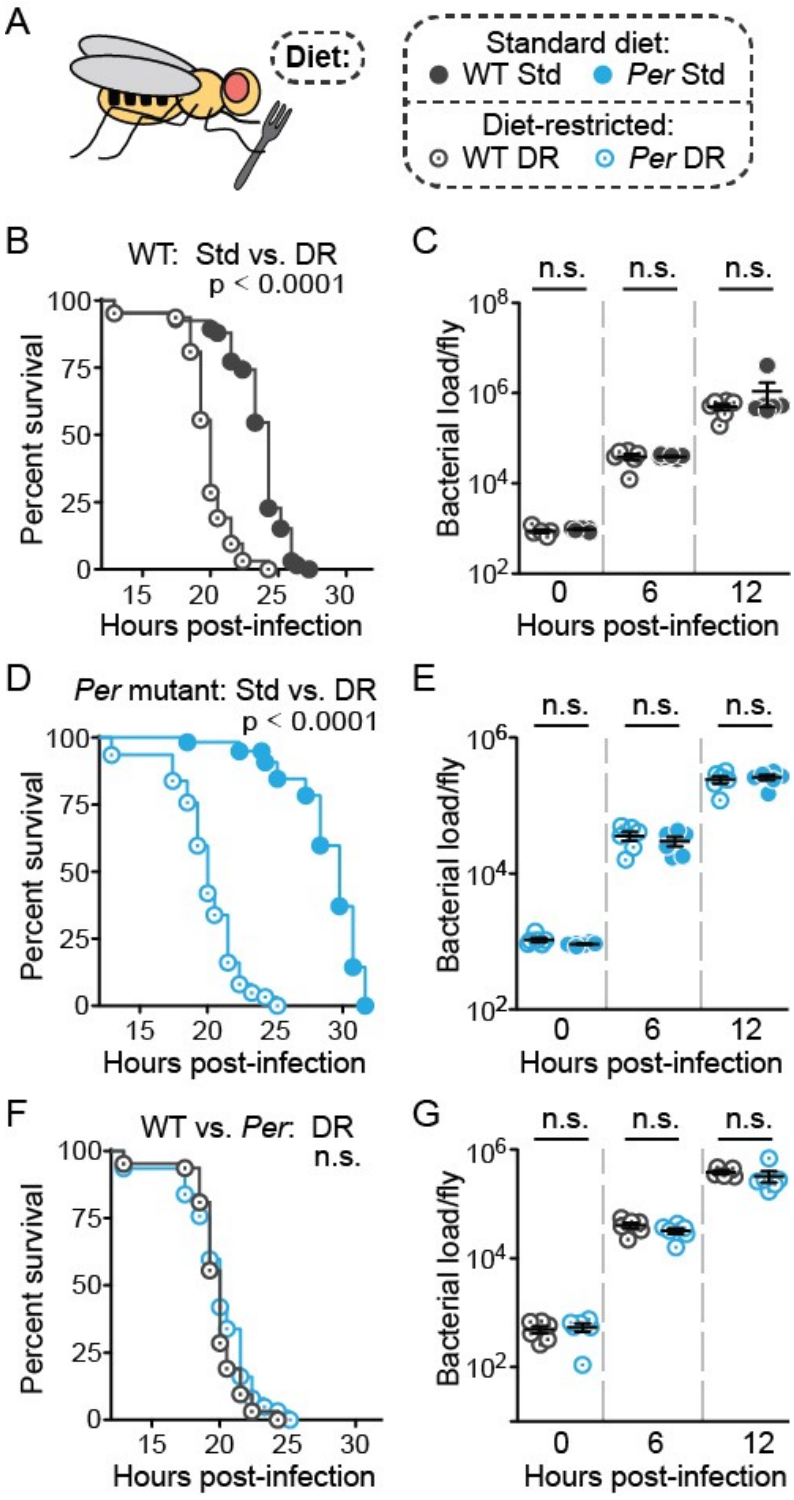

Figure 3: Dietary restriction does not increase infection tolerance of either *Per*^{01} mutants or wild type. A) Schematic of dietary conditions: wild-type flies and *Per*^{01} mutants were raised on standard food (Std) and then transferred to fresh Std food or subjected to dietary restriction on 1% glucose (DR) for 24 hours prior to and during *B. cepacia* infection. Dietary restriction decreased survival time after infection for both B) wild type (Std food n=66, DR n=63, p<0.0001) and D) *Per*^{01} mutants (Std food n=59, DR n=62, p<0.0001). F) Dietary restriction eliminated the consistent survival advantage of *Per*^{01} mutants over wild-type flies (*Per*^{01} n=62, WT n=63, n.s.). Dietary restriction did not alter bacterial load for C) wild type (n≥5 flies/time point) or E) *Per*^{01} mutants (n≥5 flies/time point, n.s., all time points); moreover, G) diet-restricted wild type and *Per*^{01} mutants had similar bacterial loads (n≥5 flies/time point, n.s.). p-values were obtained by unpaired t-test (0h) and non-parametric Mann-Whitney test (other time points). p-values for survival curve comparisons were obtained by log-rank analysis; p-values for bacterial load comparisons were obtained using unpaired t-tests for 0 hour time points, while subsequent time points were tested with non-parametric Mann-Whitney U tests. Error bars represent the mean ± S.E.M.; n.s.=not significant (p>0.05).

Dietary glucose and amino acids enhance infection tolerance in wild-type flies.

To identify specific dietary components contributing to tolerance of infection, we supplemented the

restricted diet with defined nutrients (Figure 4A). Because Per^{01} mutants display pleiotropic defects in metabolism and other circadian-regulated physiologies [3], we focused on wild-type flies. We first tested if increased dietary glucose complements the restricted diet, which contains 1% glucose, by comparing the effects of titrating dietary glucose (1%, 5%, 10%, or 15% glucose, no protein) with standard food (5-10% sugar, plus yeast extract). Wild-type flies exhibited shortest survival time when switched to 1% dietary glucose 24 hours before infection and survived longest on standard food (Figure 4B, $p<0.0001$ comparing standard food or 1% glucose with any other condition). While increasing dietary glucose from 1% to 5% increased survival time (Figure 4B, $p<0.0001$), further increases in dietary glucose did not (4B, $p>0.05$ for any pair-wise comparison of 5%, 10%, and 15% glucose). Despite the survival benefit conferred by 5% glucose relative to 1% glucose, bacterial load was unchanged (Figure 4D, $p>0.05$ for all time points). Moreover, no glucose-only diets increased survival time to that observed on standard food ($p<0.0001$). Thus glucose enhances infection tolerance, but glucose alone is not sufficient for optimal survival of infection. This result suggests that other components in standard food also contribute to survival of *B. cepacia* infection.

In addition to sugar, standard food contains a complex mixture of lipids, proteins, vitamins, and other nutrients derived from yeast and cornmeal ingredients. We tested whether 5% glucose supplemented with amino acids was sufficient to substitute for standard food. A diet of 5% glucose plus amino acids 24 hours before infection significantly increased survival time relative to 5% glucose alone (Figure 4C, $p<0.0001$, Figure S2A), with no change in bacterial load (Figure 4E, all time points $p>0.05$). In fact, 5% glucose plus amino acids was sufficient to increase survival time to that observed with standard food (Figure 4C, $p>0.05$). The survival benefit of amino acids was not dependent on high glucose and was also observed with 1% glucose diet (Figure S2B). Thus both dietary glucose and amino acids contribute to tolerance of infection, and acute exposure to both nutrients ~24 hours before *B. cepacia* infection is necessary for optimal survival.

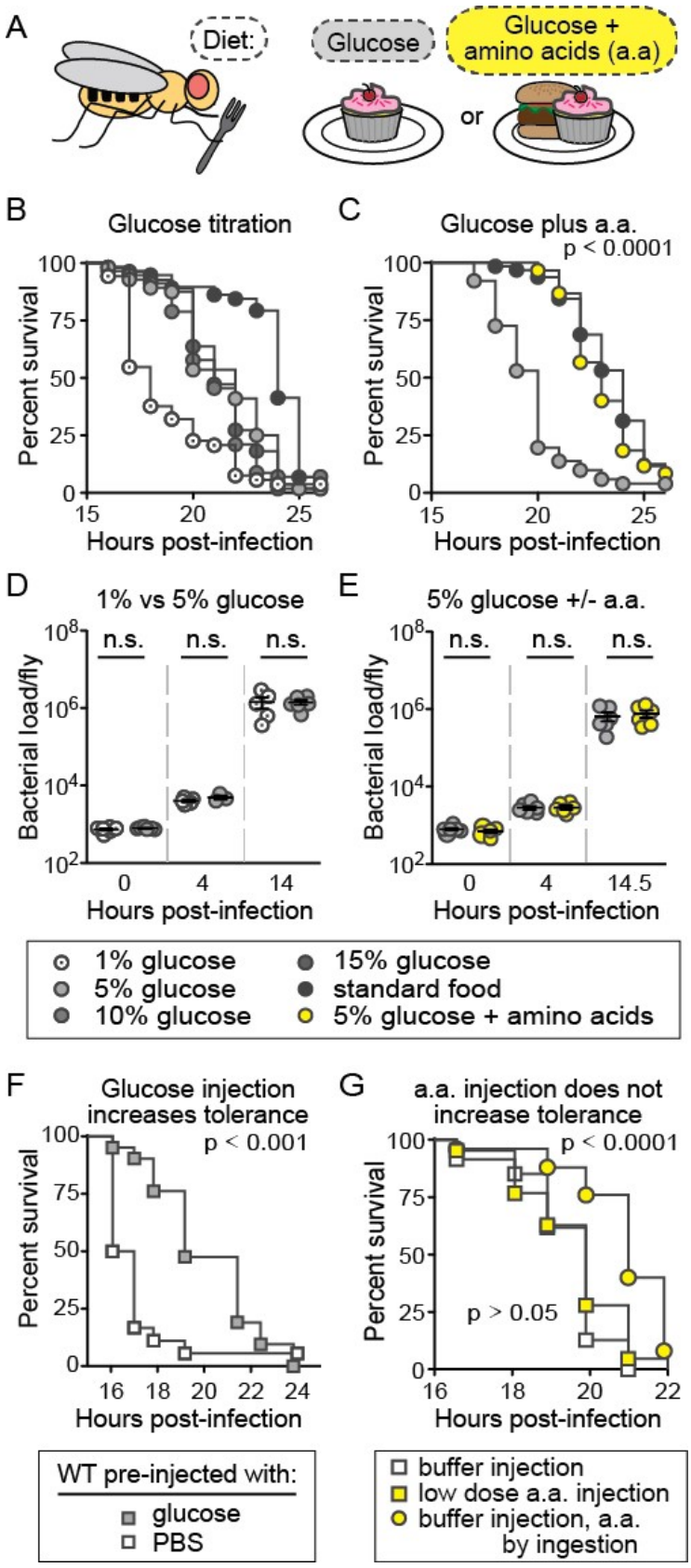

Figure 4: Glucose and amino acids increase tolerance of *B. cepacia* infection. A) Schematic of dietary conditions: wild-type flies were raised on standard food and switched 24 hours before *B. cepacia* infection to fresh standard food, glucose diets (B,D) or glucose diet plus amino acids (C,E). B) Increasing glucose concentration (5%, 10%, or 15%) increased survival time relative to 1% glucose diet ($n \geq 53$, $p<0.0001$ in all cases) and caused similar survival kinetics compared to each other ($n \geq 55$, n.s. in all cases). Flies on standard food (n=58) survived longer than flies on any glucose diet ($p<0.0001$ for all). C) Supplementing 5% glucose with amino acids (n=60) increased survival time significantly longer than 5% glucose alone (n=51, $p<0.0001$ in all cases) and was sufficient for survival kinetics similar to standard food (n=64, n.s.). There was no difference in bacterial load comparing flies fed D) 1% vs. 5% glucose (n=6 flies/time point, n.s. for all) or E) 5% glucose vs. 5% glucose plus amino acids (n=6 flies/time point, n.s. for all). F) Wild-type flies survived longer when injected 1.5 hours before infection with 50 nL of 5% glucose (n=21) than with PBS control (n=18, $p=0.0007$). G) Injection of amino acids prior to infection (n=43) does not increase the survival advantage relative to buffer alone (n=47, n.s.), and buffer injection does not eliminate the survival advantage provided by amino acid ingestion (n=25, $p<0.0001$). Additional examples of nutrient injections are shown in Figure S2. p-values for survival curve comparisons were obtained by log-rank analysis; p-values for bacterial load comparisons were obtained by unpaired t-test (0h) and non-parametric Mann-Whitney test (later time points); error bars represent the mean ± S.E.M.; a.a.=amino acids; n.s.=not significant ($p>0.05$).

Glucose is required at the time of infection for increased host tolerance.

We set out to more precisely characterize the required timing of the glucose contribution to infection tolerance. We found that a 50 nL injection of 5% glucose administered into the circulatory system of diet-restricted flies could significantly increase infection survival time relative to buffer injection (Figure 4F, p=0.0007). This dose of glucose is equivalent to the quantity ingested by a single fly in 1 hour (calculated from feeding experiments; Figure 2D-E). Glucose injection most often promoted survival when administered within 2 hours before or at the time of infection (Figure 4F, 5/8 experiments). In contrast, glucose injected more than 2 hours before infection or after infection rarely provided any survival benefit (Figure S2C-D, 1/11 experiments). Thus, with our infection protocol, the effective time window for glucose-induced survival is unexpectedly narrow, consistent with an acute rather than chronic effect of diet upon infection tolerance. These results suggest that acute glucose intake stimulates specific signaling pathways that increase immune tolerance when activated around the time of infection.

Injection of amino acids at two different concentrations at different time points before or during infection did not improve survival time (Figure 4G, amino acids vs. buffer injection, $p>0.05$; also Figure S2E-G). Flies injected with buffer were still able to respond to dietary amino acids (Figure 4G, $p<0.0001$). Thus, in contrast to glucose, amino acids appear to stimulate infection tolerance only when ingested and not when injected.

Increased TORC1 signaling correlates with increased survival for *Per^{01}* mutants and flies with greater nutrient availability.

Since transient exposure to nutrients enhances infection tolerance, we next wanted to determine whether molecular pathways stimulated by these nutrients play a role in survival of *B. cepacia* infection. The role of insulin-like signaling during infection has been characterized in *Drosophila* [153, 170-173]. We focused instead on the less well-characterized role of the kinase TOR in innate immunity, as TOR complex 1 (TORC1) is the canonical sensor of amino acid availability [160].

We first set out to determine if TORC1 kinase activity is circadian-regulated by monitoring phosphorylation of its downstream target S6K over the circadian cycle in wild type and *Per^{01}* mutants. We found that TORC1 activity oscillates over the circadian cycle in wild-type flies, with a peak of activity at

ZT9-13 (Figure 5A). This peak of TORC1 activity correlates with low Per protein levels in wild type [174]. Consistent with this, TORC1 activity did not oscillate in Per^{01} mutants and exhibited high, equivalent levels at both ZT9 and ZT21 (Figure 5B). Thus TORC1 activation is circadian-regulated and increased in Per^{01} mutants during the time course of infection, suggesting that increased TORC1 activation may contribute to Per^{01} mutants' increased survival of infection.

We next tested TORC1 activity of wild-type flies in dietary conditions associated with increased survival of infection. We found that TORC1 activity was higher in flies fed food containing amino acids than in flies fed food without amino acids (Figure 5C, all $p \leq 0.0163$). Thus both wild-type flies on nutrient-rich diets and Per^{01} mutants exhibit increased TORC1 kinase activity. Interestingly, TORC1 activity is higher in flies fed 5% glucose plus amino acids than those fed standard food ($p=0.0014$), suggesting that TORC1 activity may not solely mediate differences in survival.

Decreased TORC1 signaling causes decreased resistance.

To directly test the role of TORC1 in survival of infection, we inhibited TORC1 activity in two ways. First, we injected flies with rapamycin, a TORC1-specific inhibitor (9.6 ng per fly, equivalent to the mammalian dose of 16 mg/kg [175, 176]. Injection of rapamycin inhibited survival of infection relative to injection of buffer alone (Figure 5D, $p<0.0001$). Unexpectedly, we found that rapamycin-injected flies had increased bacterial load, indicating decreased resistance (Figure 5E, $p>0.05$, $p=0.0049$, $p=0.0198$). Second, we inhibited TORC1 activity using a temperature-driven system to over-express Tsc1 and Tsc2, proteins forming a TORC1-inhibitory complex [177]. Tsc1/2 over-expression was confirmed by qRT-PCR (Figure S3A-B). Similar to rapamycin injection, genetic inhibition of TORC1 reduced survival after *B. cepacia* infection (Figure 5F, $p<0.0001$ for both controls) and caused increased bacterial loads (Figure 5G, $p>0.05$, $p=0.0367$, $p=0.0022$). Taken together, these results suggest that in flies, as in vertebrates [178, 179], TORC1 mediates resistance against *B. cepacia* infection. While inhibition of TORC1 in Per^{01} mutants with rapamycin injection decreased their survival after infection (Figure S3C), rapamycin injection did not abolish Per^{01} mutants' survival advantage over wild-type controls (Figure S3D), suggesting that increased TORC1 activity is not solely responsible for their increased survival.

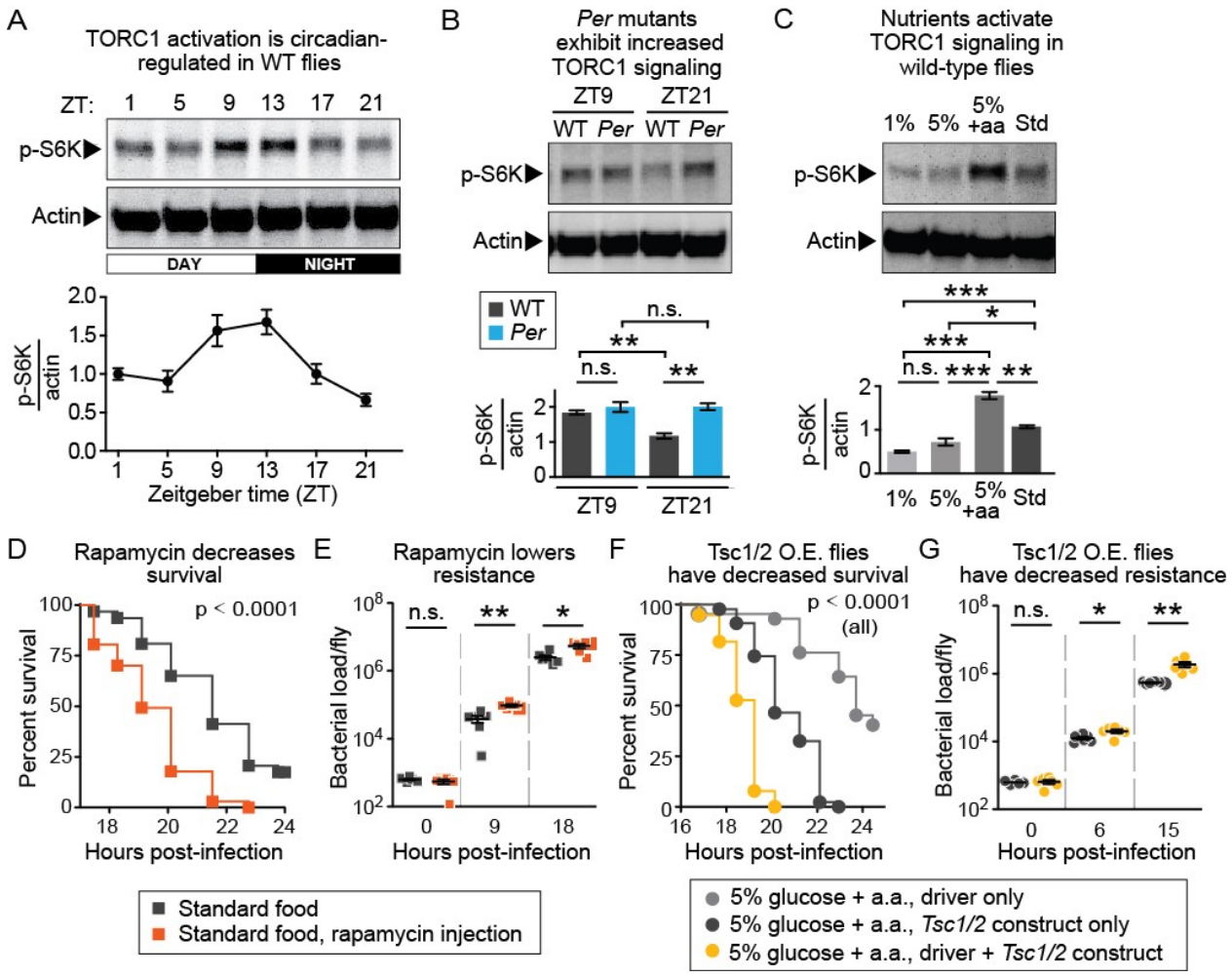

Figure 5: TORC1 signaling increases tolerance of infection. A) TORC1 activation is circadian regulated in wild-type flies: Western blot analysis (upper panel) and quantification (lower panel) showing phospho-S6K levels peak in the evening and trough in the morning. B) *Per*^{01} mutants exhibit increased levels of phospho-S6K at ZT21, as determined by Western blot analysis (n=10, ZT21 p=0.0027, ZT9 n.s.). WT flies exhibit reduced levels of phospho-S6K at ZT21 compared to ZT9 (n=10, p=0.0026). *Per*^{01} mutants do not show this difference (n.s.). C) Nutrients activate TORC1 signaling in wild-type flies, as evidenced by increased levels of phospho-S6K (n=10, p≤0.0163 for all comparisons except 1% glucose vs. 5% glucose, n.s.). D-E) Inhibition of TORC1 by co-injection of rapamycin at the time of infection reduces resistance, as shown by: D) reduced survival (n=67) compared to co-injection of buffer (n=70, p<0.0001) and E) increased bacterial load after infection (n=6, 0 hrs n.s., 9 hrs p=0.0049, 18 hrs p=0.0198). See also Figure S3C-D for infections of *Per*^{01} mutants co-injected with rapamycin. F-G) Inhibition of TORC1 by over-expression of Tsc1/2 (see also Figure S3A-B) also reduces resistance: Tsc1/2 overexpression (O.E.) mutants (n=38) exhibit F) decreased survival time relative to flies containing the driver alone (n=42, p<0.0001) or the construct alone (n=43, p<0.0001) and G) increased bacterial load after infection (n=6 for both mutant and construct alone, 0 hrs n.s., 6 hrs p=0.0367, 15 hrs p=0.0022). p-values for survival curve comparisons were obtained by log-rank analysis; p-values for bacterial load comparisons were obtained using unpaired t-tests for 0 hour time points, while subsequent time points were tested with non-parametric Mann-Whitney U tests; error bars represent the mean ± S.E.M; ZT=zeitgeber; aa=amino acids; n.s.=not significant (p>0.05); *=p≤0.05; **=p≤0.01; ***=p≤0.001.

Increased resistance is correlated with decreased TORC2 signaling.

TOR kinase associates with another, less well-understood complex, TORC2. Since TORC1 and TORC2 might compete for limited TOR kinase and these complexes appear to have opposing roles in cell growth and T cell differentiation [161, 162], we next asked whether TORC2 activity underlies infection tolerance. TORC2 is not known to play a role in survival of infection. To test this, we reduced TORC2 signaling in two ways.

First, we examined the survival of mutants lacking RicTOR, an essential molecular component of TORC2 but not TORC1, after *B. cepacia* infection [104]. *rictor*$^{\Delta 2}$ mutants had the opposite survival phenotype as that seen with TORC1 inhibition: they lived dramatically longer than isogenic controls (Figure 6A, $p<0.0001$). We also found that *rictor*$^{\Delta 2}$ mutants carried decreased bacterial load relative to wild type (Figure 6B, $p>0.05$, $p=0.0087$, $p=0.0022$). These results suggest that, while TORC1 activates resistance, TORC2 inhibits resistance.

To confirm this, we examined mutants lacking Sin1, another TORC2-specific component [180]. Similar to *rictor*$^{\Delta 2}$ mutants, *Sin1*^{e03756} mutants exhibited increased survival time after infection and decreased bacterial load relative to wild type (Figure 6C, $p<0.0001$, Figure 6D, $p>0.05$, $p>0.05$, $p=0.0043$). Thus, inhibition of TORC2 by loss of either RicTOR or Sin1 increased both survival and resistance against *B. cepacia* infection.

Because increased tolerance is defined functionally as increased survival without decreased bacterial load, increased resistance due to dietary TORC1 activation might mask increased tolerance due to genetic TORC2 inhibition. We therefore tested *Sin1*^{e03756} mutants for survival of infection and bacterial load in the absence of dietary amino acids. Consistent with TORC2 inhibition of tolerance, *Sin1*^{e03756} mutants survived infection longer than wild type with no decrease in bacterial load (Figure 6E, $p=0.0051$, Figure 6F, all $p>0.05$). Interestingly, *Sin1*^{e03756} mutants without amino acids had identical survival kinetics and bacterial load as wild-type flies fed amino acids, suggesting that amino acids had an equivalent effect on tolerance as loss of Sin1 (Figure S4A-B). These results suggest that Sin1, an essential component of TORC2, inhibits both resistance and tolerance of *B. cepacia* infection.

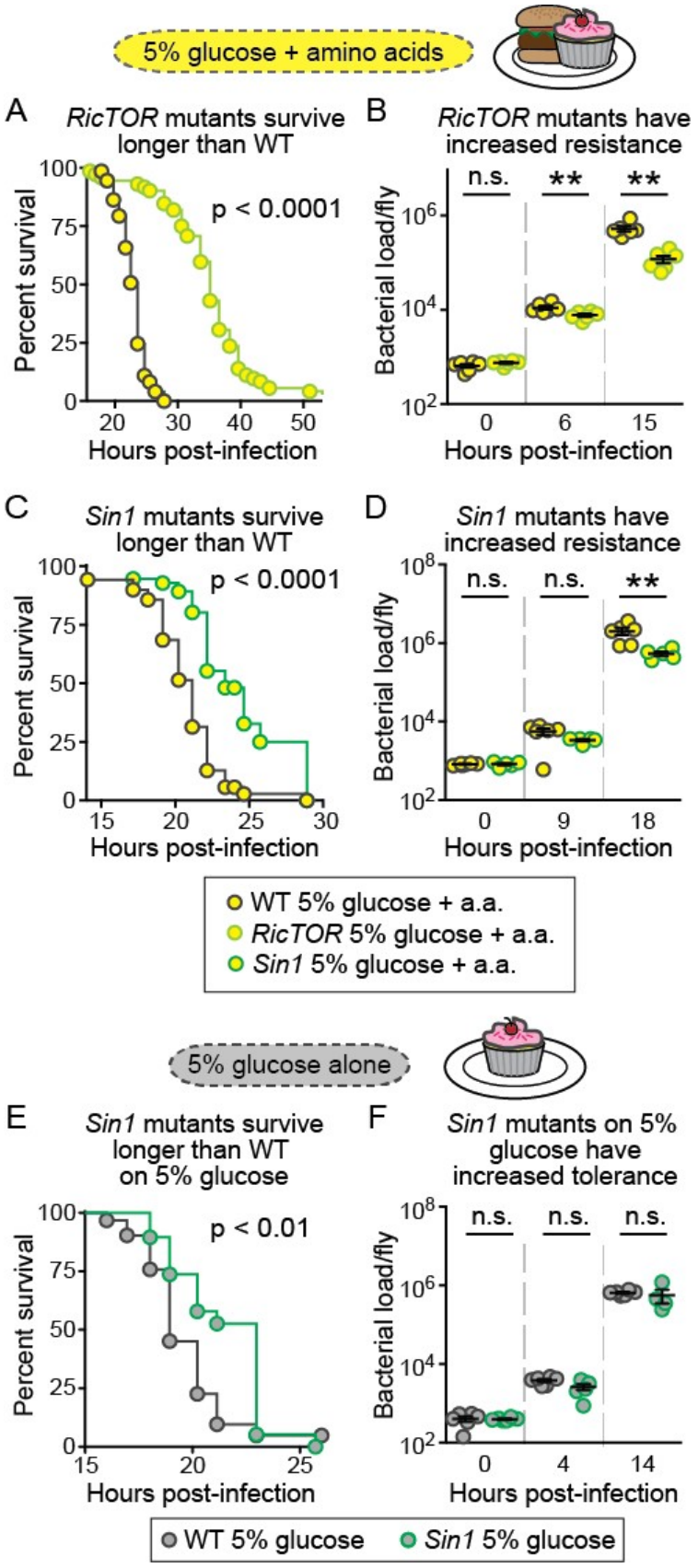

Figure 6: TORC2 activity decreases both resistance and tolerance of infection. RicTOR and Sin1 are two components of TORC2. A-B) Loss of RicTOR increases resistance in the presence of dietary amino acids, as *rictor*$^{\Delta 2}$ mutants: A) (n=72) survive infection longer than wild-type flies (n=73, p<0.0001) and B) exhibit decreased bacterial load after infection (n=6, all groups, 0 hr n.s., 6 hrs p=0.0087, 15 hrs p=0.0022). C-D) Loss of Sin1 also increases resistance in the presence of dietary amino acids, as *Sin1*^{e03756} mutants: C) (n=56) survive infection longer than wild-type flies (n=70, p<0.0001) and D) exhibit decreased bacterial load after infection (n=6, all groups, 0 hr n.s., 9 hrs n.s., 18 hrs p=0.0043). E-F) In the absence of dietary amino acids (5% glucose alone), *Sin1*^{e03756} mutants exhibit increased tolerance: E) *Sin1*^{e03756} mutants (n=24) survive infection longer than wild type (n=19, p=0.0051) and F) have similar bacterial load after infection (all n≥4, n.s.). See also Figure S4 for additional infection data for *Sin1*^{e03756} and *rictor*$^{\Delta 1/\Delta 2}$. p-values for survival curve comparisons were obtained by log-rank analysis; p-values for bacterial load comparisons were obtained using unpaired t-tests for 0 hour time points, while subsequent time points were tested with non-parametric Mann-Whitney U tests; error bars represent the mean ± S.E.M.; n.s.=not significant (p>0.05); **=p≤0.01.

Discussion

By examining a circadian mutant with increased infection tolerance against *B. cepacia,* we identified increased feeding as a circadian-regulated behavior contributing to increased tolerance. Increased feeding by Per^{01} mutants was not associated with increased energy stores, suggesting that their increased tolerance does not depend on metabolic reserves. Two specific nutrients, glucose and amino acids, fully substitute for standard food in promoting optimal tolerance after *B. cepacia* infection. Our data suggest a narrow window for glucose's contribution to survival—with this rapid infection, an increase in circulating glucose in the two hours before infection can increase overall survival time. This is consistent with the hypothesis that nutrient sensing leads to an acute activation of infection tolerance (Figure 7). Thus what and how much a fly ingests near the time of infection has a significant effect on its survival of infection.

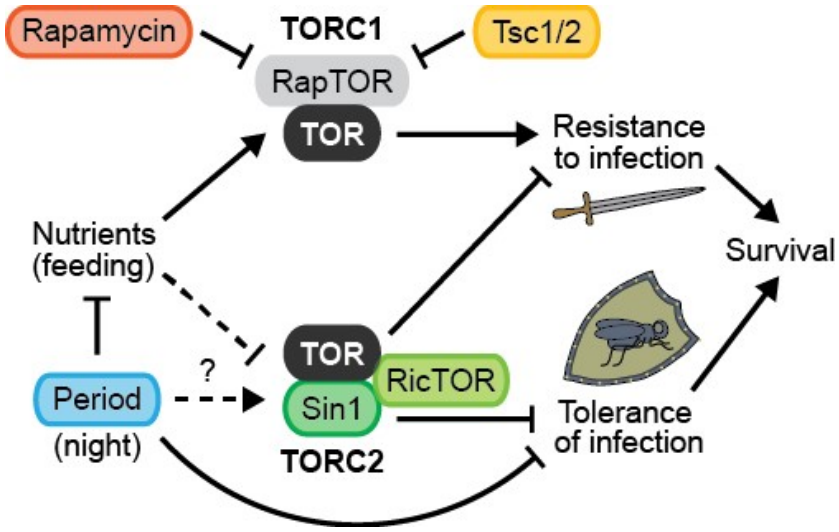

Figure 7: Schematic for nutrient-dependent and TOR signaling effects on survival from infection. Period activity decreases food (nutrient) consumption and reduces both resistance and tolerance to infection. Period and nutrients both regulate TORC1 signaling to modulate resistance to infection. Period also inhibits tolerance from infection, perhaps via promoting the tolerance inhibitory function of TORC2.

To explore the effects of dietary amino acids on survival of infection, we investigated the role of TORC1 signaling, a canonical amino acid sensing pathway. We found that TORC1 kinase activity oscillates with circadian rhythm, likely through circadian-regulated feeding behavior as seen in vertebrates [181, 182]. We also uncovered a role for TORC1 in resistance against infection in *Drosophila*. In vertebrates, TORC1 is known to mediate resistance and rapamycin is a well-characterized immunosuppressant; however, these immunosuppressive effects are thought to result primarily from

inhibiting the growth and maturation of dendritic cells and T-cells [109], adaptive immune cell types with no clear functional analogs in *Drosophila*. Our data now suggest a role for TORC1 in innate immunity against infection (Figure 7). It remains to be seen whether rapamycin acts as an immuno-suppressant for *Drosophila* infected with other pathogens besides *B. cepacia*. These results potentially open the genetically tractable system of *Drosophila* to investigating TORC1 interactions with innate immune components.

We further found a novel role for the less well-known TOR complex 2 as a potent inhibitor of immunity—that is, loss of TORC2-specific components RicTOR or Sin1 caused dramatic increases in survival time after infection and impacted both resistance and tolerance (Figure 7). Loss of Sin1 increases resistance in the presence of amino acids and increases tolerance in the absence of amino acids. Because there exists a resistance phenotype, possibly due to amino acids-stimulation of TORC1, we cannot say whether loss of Sin1 increases tolerance in the presence of amino acids, as host tolerance is functionally defined as changes in survival in the absence of correlated changes in bacterial load. *rictor*$^{\Delta 2}$ mutants in the presence or absence of amino acids exhibit increased resistance to infection (Figure S4C-D). The disparity between *Sin1*^{e03756} and *rictor*$^{\Delta 2}$ mutants could be due to differences in the distribution of TOR between TORC1 and TORC2 lacking one component or the other. Our results suggest that TORC1 and TORC2 act in opposition during immunity and we speculate that these complexes may be oppositely circadian-regulated--that is, *Per* mutants have high TORC1 and low TORC2 activity.

The finding that TORC2 inhibition increases survival of infection is surprising but not completely without precedent. TORC2 is mainly thought to play a role in tissue-specific morphology, stimulated by growth factors and PI3K and acting on downstream targets such as cytoskeletal components, Akt, and SGK1 [106, 175, 183]. In *Drosophila*, TORC2 has been implicated in tolerance of heat stress [184], cell and tissue growth [185, 186], and neuronal outgrowth [187, 188]. While most immune effects of TOR are thought to act through TORC1, recent evidence suggests that, in mouse embryonic fibroblasts, RicTOR inhibits Toll-like receptor-stimulated cytokine expression [189]. Thus RicTOR may have conserved immune-suppressive effects in both vertebrates and invertebrates. While the direct targets of TORC2 relevant for infection resistance and tolerance remain unknown, their identification will be an important goal of future studies.

The cellular and molecular mechanisms that promote host tolerance of infection are not well-understood [20, 150]. *B. cepacia* is a significant opportunistic bacterial pathogen, particularly in hospital settings with susceptible patients [159]. This hospital-acquired infection can be associated with high rates of mortality, up to 50% for severe strains, and is often antibiotic-resistant [190, 191]. Understanding the tolerance mechanisms stimulated by acute glucose and dietary amino acids will help to identify targets for pharmacological treatments. Here we have identified TORC2 as a potential pharmacological target to increase host survival time after infection, as TORC2 mutants are able to survive infection up to 59% longer than wild type. The potential therapeutic value of TORC2 inhibition has not been explored, as there are currently no known small molecule inhibitors specific to TORC2 and not TORC1. The *Drosophila* model of infection described here may therefore prove useful in screening for such TORC2-specific inhibitors and for further dissection of acute, nutrient-stimulated, TOR-mediated host defenses against bacterial infections such as *B. cepacia*.

Methods

(See Appendix 1 for details.)

Fly strains

$w^{1118}per^{01}$ (null) mutants [174] were outcrossed with a w^{1118} Canton S strain, used as isogenic controls [192]. Wild-type Oregon R flies were used to test effects of dietary components and rapamycin. *UAS-Tsc1/Tsc2* (from Marc Tatar [193]) homozygous males were crossed to w^{1118}*;tub>Gal80-ts;tub>Gal4/TM6c* virgins and maintained at 18ºC until 29ºC transgene induction 24 or 48 hours before infection. *rictor* null mutants (imprecise p-element excision alleles $rictor^{01}$ *and* $rictor^{02}$) and precise excision controls were obtained from Stephen Cohen [186]. Experiments used hemizygous $rictor^{02}$ flies crossed to $rictor^{01}$. $Sin1^{e03756}$ (SAPK-interacting protein 1) mutants are null piggyBac transposon insertion mutants from Bloomington *Drosophila* Stock Center, stock #18188 [186]. 5-10 day-old males raised on standard molasses food were used for all experiments.

Infections

Infections were performed as described [164] with *Burkholderia cepacia* (ATCC strain #25416). Death was assayed visually the next day every hour or more frequently as needed. Survival curves are plotted as Kaplan-Meier graphs and log-rank analysis performed using GraphPad Prism. All infection experiments were performed with a minimum of 3 independent trials and yielded statistically similar results, except where noted. Graphs and p-values in figures are representative trials.

Bacterial load quantitation

Bacterial load was quantified as described [164] and analyzed by unpaired t-tests for 0 hour time points; subsequent time points were analyzed with non-parametric Mann-Whitney tests, which does not assume normal distribution as bacteria grow exponentially. Data are plotted with SEM.

qRT-PCR, melanization, and phagocytosis assays

Assays were performed as described, using *B. cepacia* for infection [19, 146]. p-values for AMP induction and melanization were obtained by t-tests for three independent trials; data are represented as mean ± SEM. p-values for phagocytosis assays were obtained by log-rank analysis. See Supplement for primer sequences.

Starvation assay

Using the DAM5 system (TriKinetics), 5-7 day-old male flies were incubated on agar alone. Time of death was determined by complete loss of movement. p-values were obtained by log-rank analysis.

Metabolic storage assays

Samples consisted of eight male flies (5-10 days old) homogenized in buffer. Metabolic storage levels were measured by enzyme-based colorimetric assays as described [153, 194]. Values were normalized to the average weight for that genotype and to the mean value for wild type, then plotted with the normalized SEM. p-values were obtained by unpaired t-test.

Feeding assays

CAFE assays and ^{32}P feeding assays were performed as described [167, 168, 195]. p-values were obtained by unpaired t-test; data are represented as mean ± SEM.

Protein extraction and Western blotting

Western blot analysis of whole-fly homogenates was performed by standard methods using 1:1000 anti-phospho-S6K (Thr398) (Cell Signaling #9209), 1:10,000 anti-Actin-HRP (Sigma **A3854**), and 1:2000 anti-rabbit-HRP (Cell Signaling #7074). p-values were obtained by unpaired t-test; data are represented as mean ± SEM.

AUTHOR CONTRIBUTIONS

MSH, VWA, RMO, and MJU designed experiments. Experiments were performed by: VWA, RMO, and CGZ (survival, bacterial load); VWA (AMPs, starvation); RMO (metabolic storage); MJU (Western blots); EFS (phagocytosis); VMH (melanization); KRM and WWJ (feeding). MSH, JCC, VWA, and RMO produced the manuscript.

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III. The role of circadian regulation in immunosenescence

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Abstract

Elderly patients experience a higher rate of mortality from infections, especially pneumonia and sepsis. However, it remains unknown precisely how aging affects innate immune function. Studying age-related effects on the mammalian innate immune system is complicated by crosstalk between innate and adaptive immune signaling pathways, as well as the expense of maintaining animals during the lengthy aging process. Fruit flies' lack of an adaptive immune system, conserved innate immune components, and comparatively brief lifespan make them an ideal model organism for studying how age affects innate immunity.

We show that age reduces functional immunity to specific bacterial infections. We present evidence that both the ability to kill and clear bacteria, and the ability to survive the pathogenic effects of infection, are reduced in old flies. Finally, we demonstrate that phagocytosis, a specific immune mechanism, is reduced in old flies during part of the circadian cycle. These findings suggest that precise innate immune mechanisms become dysregulated with age, and that immunosenescence may be related to the decline in robust circadian function that is observed during aging in many animals. Ultimately, our work contributes new information to the growing body of knowledge about immunosenescence, which will be important as human lifespan increases worldwide.

Introduction

The number of people aged 65 and over in the U.S. is expected to double between 2012 and 2050 [34], creating an urgent need to improve healthcare for seniors. Aging is clearly a risk factor for many diseases including bacterial infection [36]. However, the underlying mechanistic reasons for disease susceptibility are not well-defined in many cases. Aging and innate immunity are evolutionarily conserved properties of mammals and invertebrates. Therefore, studying immunosenescence in a short-lived, genetically tractable model system like *Drosophila* could yield important insights.

In both *Drosophila* and vertebrates, the innate immune system exhibits altered function with age. Previous studies in *Drosophila* have demonstrated that aging may reduce survival of infection in flies [82-84], but these studies were limited to younger flies or bacteria that are not typically pathogenic. It remains to be determined how advanced age affects survival of pathogenic infections.

Two types of immunity affect survival of bacterial infection, resistance and tolerance. Resistance is the ability to survive infections by directly limiting microbial growth. Tolerance is the ability to survive the infections by limiting the pathogenic effects of infection. We distinguish between these two physiologies by quantifying bacterial load. Longer survival coupled with a reduced bacterial load indicates improved resistance, while longer survival despite a similar bacterial load indicates improved tolerance. Although some *Drosophila* studies included bacterial load assays, it's still unclear whether advanced age affects tolerance or resistance towards pathogenic infections.

Phagocytosis is an important mechanism for resistance against microbial infection in *Drosophila*. Two studies have examined senescence of the phagocytic response in *Drosophila* [92, 93]. The first found that older females had fewer circulating hemocytes than younger females, but this was not true for older males [92]. In the latter study, destruction of the phagocytosed microbe was shown to be slowed in older phagocytes [93]. Interestingly, these studies gave conflicting results in their analysis of whether the proportion of phagocytically active hemocytes declines with age. This underscores the need for further study of how aging affects phagocytosis in *Drosophila*.

Circadian proteins regulate innate immunity in *Drosophila* [19, 146], including the phagocytic activity of immune cells [92, 196]. Moreover, survival of specific bacterial infections is also circadian-regulated [142]. Similar to aging in humans, aging of flies is associated with circadian dysregulation in

several ways: lengthened free-running period of locomotor activity rhythms, reduced levels of core circadian clock proteins Per and Tim, and dampened expression of Clk-Cyc transcriptional targets in fly heads [197]. Because immunity is circadian-regulated in young flies and because aging causes loss of circadian regulation, we hypothesized that aging-associated circadian dysregulation contribute to aging-associated immunosenescence.

Here we confirm that *Drosophila* undergo functional immunosenscence in their ability to survive specific bacterial infections, and exhibit both reduced tolerance and reduced resistance. We also show that phagocytosis is reduced in old flies at night, but not during the day, demonstrating a reduction in circadian-regulated immune system function with age. We found that *Period* circadian mutants experience similar immunosenescence compared to wild-type flies. Finally, it was recently shown that over-expression of the circadian protein *cryptochrome* (*cry*) in aged flies can restore circadian regulation in older flies and improve healthspan, specifically resistance of oxidative stress [198]. Thus we set out to test whether *cry* overexpression in aged flies and concomitant extension of normal circadian regulation also extends healthspan regarding phagocytosis by immune cells and survival of bacterial infection.

Results

Old flies are more susceptible to specific pathogens.

We wanted to test whether like old people, old flies are more susceptible to certain pathogens. Therefore, we infected old and young flies with a variety of pathogens well-characterized in *Drosophila* infections, including *Streptococcus pneumoniae*, *Listeria monocytogenes*, and *Pseudomonas aeruginosa* [19, 123, 199]. We found that aged flies were more sensitive to infection by *S. pneumoniae* (Figure 1A) and *L. monocytogenes* (Figure 1B). In contrast, aged flies survived infection by *P. aeruginosa* similarly to young flies (Figure 1C). This suggests that flies undergo functional immunosenescence, and that aging limits their ability to fight specific bacterial infections.

Old flies show both reduced resistance and reduced tolerance.

Two broad categories of defense against bacterial infection are resistance, control of microbial growth, and tolerance, control of the pathogenic effects of infection. In order to find out which of these is reduced

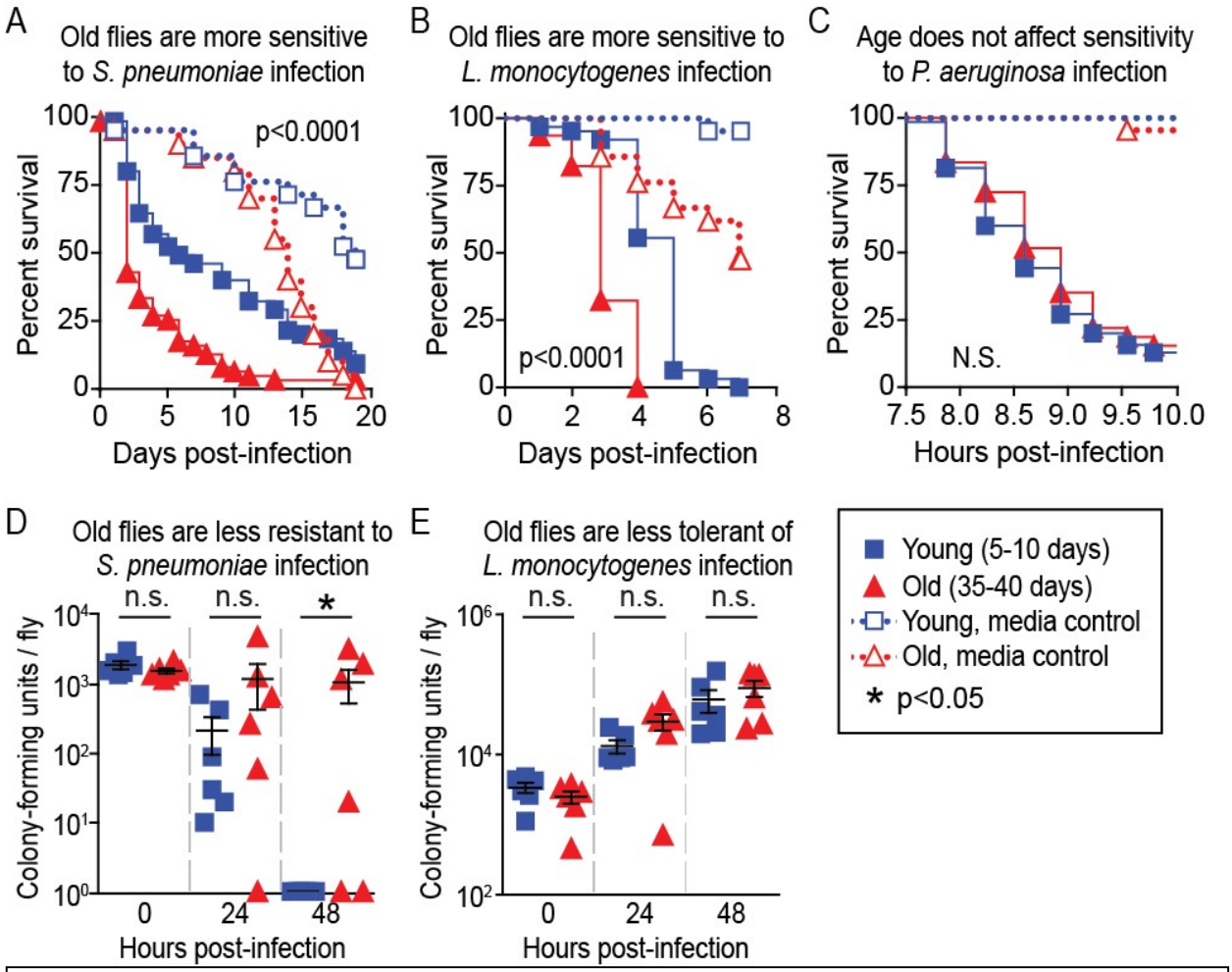

Figure 1. Old flies are more susceptible to specific pathogens. Young flies (blue) survived longer than old flies (red) when infected with (A) *S. pneumoniae* (young, n=64; old, n=63; p<0.0001) or (B) *L. monocytogenes* (young, n=63; old, n=62; p<0.0001), but not (C) *P. aeruginosa* (young, n=70; old=91; n.s.). Approximately 20 old and young flies were injected with sterile media in order to control for the effects of wounding (dotted lines). In all cases, media controls survived longer than infected flies (all, p<0.05). Old flies have increased bacterial load when infected with (D) *S. pneumoniae* (all n≈6, n.s., n.s., p=0.0284), but not when infected with (E) *L. monocytogenes (all n*≈6, all n.s.). p-values for survival curve comparisons were obtained by log-rank analysis; p-values for bacterial load comparisons were obtained using unpaired t-tests for 0 hour time points, while subsequent time points were tested with non-parametric Mann-Whitney U tests. Error bars represent the mean ± S.E.M.; n.s. = not significant (p>0.05).

by age, we assayed bacterial loads in flies infected with *S. pneumoniae* and *L. monocytogenes*. We found that when infected with *S. pneumoniae*, aged flies had increased bacterial loads relative to young flies. However, young and old flies had similar bacterial loads when infected with *L. monocytogenes*. These results, combined with old flies' reduced survival, suggest that old flies have reduced resistance to *S. pneumoniae* infection and reduced tolerance to *L. monocytogenes* infection.

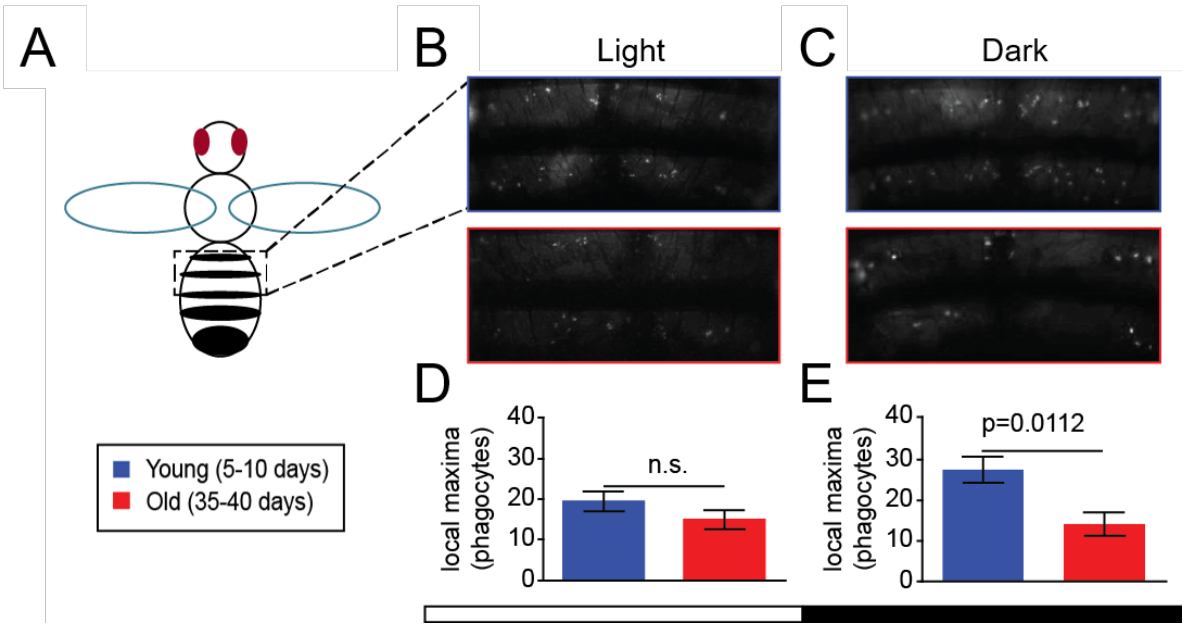

Figure 2. Old flies show reduced phagocytic ability during the dark phase, but not during the light phase. Flies were injected with dead *S. aureus* labeled with a fluorophore, then injected with quench. Only bacteria that have been phagocytosed remain fluorescent. (A) Dashed lines indicate the region enlarged in the inset. Shown here are images of the dorsal surface of representative flies during the day (B) and night (C). Phagocytic activity for young and old flies was quantified by measuring the number of local maxima. (D) Young and old flies showed similar levels of phagocytic activity during the circadian day phase (young, n=8; old, n=9; p=0.2043). (E) Young flies showed higher levels of phagocytic activity during the circadian night phase (young, n=7; old, n=6; p=0.0112). p-values were obtained by unpaired, two-tailed t-test.

Old flies show reduced phagocytic ability during the dark phase, but not during the light phase.

Phagocytosis is one resistance mechanism known to be necessary for defense against *S. pneumoniae* infection [19]. Furthermore, phagocytosis is circadian-regulated in *Drosophila* [146]. Therefore, we assayed how age impacts phagocytic ability by injecting fluorescently labeled bacteria at different circadian times (Figure 2A). We found that for flies oppositely entrained to a 12:12 LD/DL cycle, aged flies showed reduced phagocytic ability when assayed during the dark portion of the cycle (Figure 2B-D). In contrast, aged flies did not show reduced phagocytic ability when assayed during the light portion of the cycle (Figure 2E-G).

Aging does not alter a circadian mutant phenotype; a circadian mutation does not alter an aging phenotype.

Circadian *Per* mutants show reduced survival of *S. pneumoniae* infection (3A), as do old wild-type flies compared to young (Figures 1A, 3B). Both *Per* mutants and old wild-type flies also show circadian arrythmicity [197]. We hypothesized that loss of circadian regulation is the main reason that old flies are susceptible to *S. pneumoniae* infection. If this were true, we would expect *Per* mutants to have similarly poor survival whether old or young, since all *Per* mutants lack circadian regulation. Additionally, we might expect that old *Per* mutants survive similarly to old isogenic controls, since both lack circadian regulation. We infected young and old *Per* mutants and isogenic controls. Unexpectedly, we found that old wild-type flies did survive longer than old *Per* flies (Figure 3C) and that young *Per* flies did survive longer than old *Per* flies (Figure 3D). These results suggest that loss of circadian regulation is not the main driver of immunosenescence.

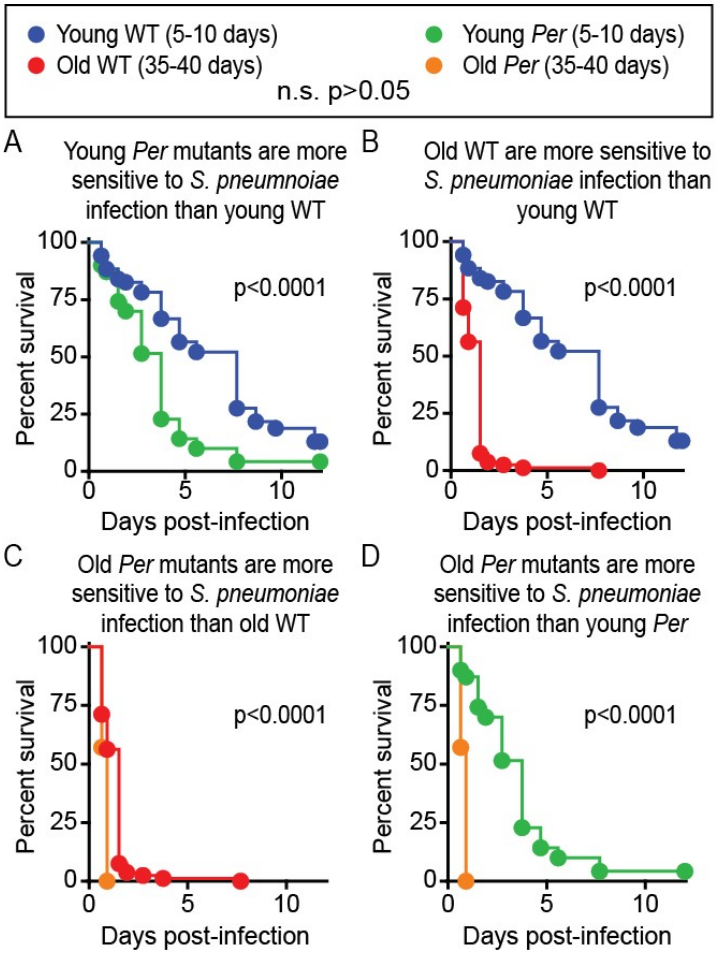

Figure 3. Per mutants undergo immunosenescence and are sensitive to *S. pneumoniae* infection whether old or young. (A) Young Per mutants (n=70) are more sensitive to *S. pneumoniae* infection than isogenic controls (n=69, p<0.0001). (B) Old wild-type flies (n=80) are more sensitive to *S. pneumoniae* infection than young wild type (n=69, p<00001. (C) Old Per mutants (n=77) are more sensitive to *S. pneumoniae* than isogenic controls (n=80, p<0.0001). (D) Old Per mutants (n=77) are more sensitive to *S. pneumoniae* infection than young Per mutants (n=70, p<0.0001). P-values for survival curve comparisons were obtained by log-rank analysis.

Does overexpression of *cryptochrome* increase immunity to *S. pneumoniae*?

For several reasons, we wanted to test whether restoring circadian regulation in old flies would increase their ability to survive *S. pneumoniae* infection. First, circadian regulation has been implicated in immunity [20, 146]. Second, circadian regulation has been implicated in survival of *S. pneumoniae* infection [20](3A). Third, old flies lose both circadian regulation [197] and the ability to survive *S. pneumoniae* infection (1A; 3B). Overexpression of *UAS-cry* under the control of the *Tim* promoter fused to Gal4 has been shown to restore circadian locomotor activity in *Drosophila* [198]. Therefore we infected old *cry* overexpressing flies and isogenic controls with *S. pneumoniae*.

Results from the infections were inconsistent, but we did observe a difference in survival between our media-injected wounding controls. In two out of three trials, *UAS-cry* isogenic controls succumbed sooner than both the *tim-Gal4* control and the *cry*-overexpressing flies (Figure 4B). This difference was not observed in any of three trials in young flies (data not shown). We hypothesized that the wounding differences in old flies might indicate a shorter lifespan in general. To test this, we assayed lifespan in the flies expressing *cry* and isogenic controls. Surprisingly, we found that *UAS-cry* controls lived longer than either of the other genotypes (Figure 4A). We concluded that attempting to rescue immunosenescence using these *Gal4-UAS* constructs would not be straightforward, and that another method to rescue circadian regulation would be necessary.

Discussion

Here we show that *Drosophila* undergo functional immunosenescence in advanced age, reducing their ability to survive specific, pathogenic bacterial infections. These results are consistent with previous studies showing that *Drosophila* undergo immunosenescence toward non-pathogenic bacteria [82] and that age can reduce survival of bacterial pathogens in relatively young flies [83]. Surprisingly, although old flies were susceptible to both *L. monocytogenes* and *S. pneumnoiae* infection, they were not susceptible to *P. aeruginosa* infection. Further studies should clarify why old flies are sensitive to some pathogenic bacteria but not others. Either different modes of bacterial pathogenesis, different host responses to infection, or some combination of the two could be responsible for the varied phenotypes we observed.

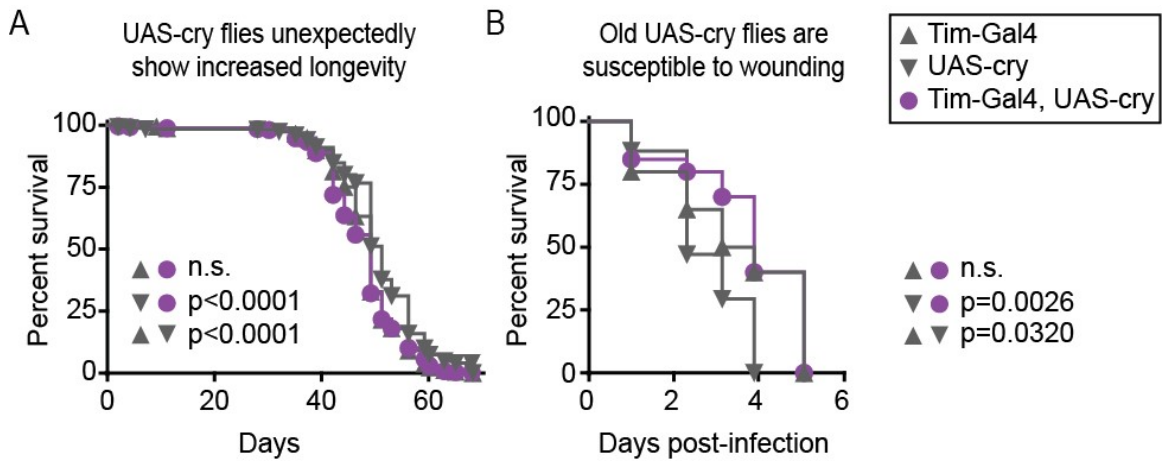

Figure 4. The UAS-cry transgene affects longevity and wounding. (A) *UAS-cry* flies show increased longevity compared to *Tim-Gal4* flies ($p<0.0001$) or to *Tim-Gal4* x *UAS-cry* flies ($p<0.0001$). n=300 for each group. (B) Old UAS-cry flies are susceptible to wounding compared to Tim-Gal4 flies ($p=0.0320$) or compared to Tim-Gal4 x UAS-cry flies ($p=0.0026$; n=20 each group).

Our data also shows that age can reduce both resistance and tolerance to infection. This is significant because tolerance is not currently well understood. *L. monocytogenes* infection in differently aged flies could therefore prove to be a useful model system for elucidating the molecular mechanisms that contribute to tolerance. The finding of both reduced resistance and reduced tolerance in old flies also indicates that multiple immune functions are subject to age-related pathologies and underscores the importance of research in this area.

We found that phagocytosis is reduced in old flies during their dark phase, or subjective night. Our data are consistent with other studies which have indicated that specific changes in phagocytic activity can result from age [92, 93]. Most notably, the first study found that the proportion of phagocytically active cells declines with age, using *E. coli* and *B. bassiana*. This is consistent with my observation that there were fewer phagocytic inclusions in the older flies compared to the younger flies. In contrast, the 2014 study observed more phagocytic inclusions per active phagocyte in older flies, and showed that this was due to a lag in phagocytic turnover. We did not measure inclusions in single phagocytes, we performed our experiments at a different circadian time, and we used males rather than virgin females, any of which could account for differences in our results.

Surprisingly, we found that phagocytosis was reduced in old flies during their dark phase, or subjective night, but not during their light phase, or subjective “day”. This may be the first description of circadian-regulated immunosenescence. Future research should identify the molecular mechanisms by

which circadian rhythm influences phagocytosis, and whether expression or activity of the relevant proteins is altered by age.

We found that *Period* circadian mutants experience similar functional immunosenescence compared to wild-type flies. This suggests that circadian regulation is not the only factor that contributes to immunity. Disappointingly, analysis of flies over-expressing *cry* did not yield consistent results. This could be due to heterogeneity in their genetic make-up, and might be fixed by back-crossing these flies again for several generations.

Here I show that aging *Drosophila* undergo functional immunosenescence in their ability to survive specific, pathogenic bacterial infections. I also show that old flies have both reduced tolerance and resistance, and that phagocytosis is reduced in old flies during the circadian night, but not day. These observations indicate that *Drosophila* will be an important model system for studying immunosenescence with the possibility of translational importance for the ever-growing population of aged humans.

Methods

Fly strains

Wild-type Oregon R flies were used to test survival, bacterial load, and phagocytosis. For experiments on *Period* mutants, $w^{1118}per^{01}$ (null) mutants [174] were outcrossed with a w^{1118} Canton S strain, used as isogenic controls [192]. Similarly, for *cry* expression experiments, *tim-Gal4* and UAS-cry^{24} were each separately crossed to a w^{1118} Canton S strain, which they had been isogenized to previously [198]. These were used as isogenic controls for flies carrying both the *tim-Gal4* and UAS-cry^{24} transgenes. Young flies were 5-10 day-old males and old flies were 35-40 day-old males for all experiments.

Fly food

Flies were bred on standard food containing cornmeal, molasses, agar, and yeast, with a total concentration of 5-10% sugar and 2-3% yeast. The mixture was boiled for 25 minutes, then let cool for 45 minutes, or until the temperature reached 65°C or less. Methylparaben (Tegosept, 50 g in 300 mL ethanol) and 180 mL of propionic acid were then added as antimicrobial agents. To allow mating, males were not separated from females until ~24 hours later to allow mating. Flies were raised at 25°C, 55–65%

humidity with a 12h:12h light:dark cycle in a Darwin Chambers incubator after collection. Flies for *cry* over-expression experiments were maintained on molasses food, with new vials provided every 3-4 days. Flies for wild type and *Per* experiments were maintained on dextrose food, with new vials provided every 7 days. Dextrose food contains cornmeal, agar, yeast, dextrose, and methylparaben, prepared similarly to standard food.

Bacterial cultures

Listeria monocytogenes (strain 10403s) was grown overnight at 37°C in 5 mL standing Brain Heart Infusion (BHI, Teknova) and diluted to an OD_{600} of 0.10 for injection into flies. *Streptococcus pneumoniae* (serotype 2) [200] was grown overnight at 37°C in tryptic soy broth to an OD_{600} of 0.40 (log phase), then frozen down to -80°C with 5% glycerol added. For infections, an aliquot was thawed, centrifuged to remove the glycerol media, and the pellet was re-suspended in BHI. For wild type infections, the thawed bacteria was then allowed to recover for 1-2 hours at 37°C. For all other infections, the thawed bacteria was immediately diluted to an OD_{600} of 0.05-0.10 for infections for wild-type flies, 0.05 for infections of *Per* mutants, and 0.03-0.05 for infections of *tim-Gal4* and UAS-*cry* constructs. *Pseudomonas aeruginosa* (strain PA14, gift of Man Wah Tan) was grown overnight at 37°C in 5 mL standing BHI and diluted to an OD_{600} of 1.0 for injection into flies.

Injections

Infections were performed as described [19]. Injections were performed with age-matched, mated males, in vials containing approximately 20 flies. For injections, flies were lightly anesthetized with CO_2. Injections were carried out with a 10 µL Drummond Scientific glass capillary needle (#3-000-210- G), machine-pulled by a Sutter Instrument Co. machine (Model P-30). A custom-modified Tritech microinjector was used to inject 50 nL of liquid into each fly. Volume was calibrated by measuring the diameter of an expelled drop in halocarbon oil 700 (Sigma, H8898) under a layer of mineral oil (Sigma, M8410). All injections were performed between (Zeitgeber time) ZT 5 and ZT 8 to minimize variability from circadian effects on immunity.

Infections

Infections were performed with *L. monocytogenes, S. pneumoniae, or P. aeruginosa*. Death was assayed visually daily or twice daily for *L. monocytogenes* and *S. pneumoniae* infections, and assayed every 15 minutes for *P. aeruginosa* infections. Survival curves are plotted as Kaplan-Meier graphs and Mantel-Cox log-rank analysis performed using GraphPad Prism. All infection experiments were performed with a minimum of 3 independent trials and yielded statistically similar results, except where noted. Graphs and p-values in figures are representative trials.

Bacterial load quantitation

Bacterial load was quantified as described [164] and analyzed by unpaired t-tests for 0 hour time points; subsequent time points were analyzed with non-parametric Mann-Whitney tests, which does not assume normal distribution as bacteria grow exponentially. Data are plotted as mean ± SEM.

Phagocytosis assays

Phagocytosis assays were performed as described in [146]. Briefly, 6-12 male flies were injected with 50 nL of 20 mg/ml FITC or pHrodo-labeled *S. aureus* or *E. coli* in water (Molecular Probes, cat# A10010 and P35361). The flies were allowed to phagocytose the particles for 30–60 min, and then the dorsal surfaces were superglued to glass coverlslips. Flies in the dark phase were at approximately ZT 19; flies in the light phase were at approximately ZT 7. Flies were maintained in 2 separate incubators with anti-phase 12h:12h light:dark cycles so as to avoid circadian effects on the experimenter.

Imaging

Fluorescence images were taken of the dorsal surface using epifluorescent illumination with a Nikon E800 microscope and Photometrics CoolSNAP HQ^2. Images were captured with NIS Elements (Nikon) software, 100-200 millisecond exposure times.

AUTHOR CONTRIBUTIONS

MSH and VWA designed experiments. Experiments were performed by SFM (ectopic *cry*

overexpression), AC (ectopic *cry* overexpression) and VWA (other). VWA and MSH produced the manuscript.

IV. Reduced Grooming is a Conserved Sickness Behavior in *Drosophila*.

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Abstract

Sickness behavior refers to a coordinated set of animal behaviors including lethargy, depression, anorexia, and reduced grooming, which are induced by systemic infection in endotherms. Although sickness behavior has been well documented in mammals, it is unknown to what degree it is conserved in insects. Nor is it known whether sickness behaviors are adaptive responses that help the host animal survive, as opposed to maladaptive consequences of debilitation that confer no survival benefit. Standardized assays for conserved sickness behaviors in a genetically tractable model organism are needed. These would allow for mutant screens that could yield important insights about whether advantages are conferred by sickness behaviors.

To this end we introduce a new method that can automatically quantify *Drosophila* grooming behavior, and we show that reduced grooming behavior is a conserved sickness behavior in fruit flies systemically infected with *L. monocytogenes*. This work establishes *Drosophila* as a model system for answering questions about reduced grooming as a sickness behavior, including whether the host benefits from reduced grooming. Future research using this model will contribute to our understanding about how animal behavior evolved and how it factors into the continuous evolutionary arms race between hosts and pathogens.

Introduction

Sickness behavior refers to a coordinated set of behaviors including lethargy, depression, and anorexia, which manifests in animals infected with bacteria, viruses, or protozoa [110]. Reduced grooming is another component of sickness behavior that is conserved among vertebrates. Drosophila are an excellent model system for studying infection and sickness behaviors including anorexia [94] and loss of circadian regulation [19]. Although reduced grooming has been a known component of mammalian sickness behavior for many decades, it remains unclear whether *Drosophila* exhibit this aspect of sickness behavior.

Studying whether *Drosophila* undergo sickness-associated loss of grooming has been difficult for several reasons. Monitoring grooming behavior requires labor- and data-intensive analysis. Grooming is a complex and subtle behavior, with two types of movement described in the fruit fly: the rubbing of legs

against each other, and the sweeping of legs over various other body parts. Until recently, most grooming data had to be obtained by human-eye video analysis. Although video methods are being developed, there are additional complications. Grooming would ideally be tracked in multiple flies for relatively long periods of time over the course of infection. Previous types of video analysis were limited to tracking only one or two individuals for short time frames, or for specific time points during infection [133, 139, 201, 202]. Existing automatic grooming tracking methods also lack detailed verification of the results. Because of these limitations, most groups must still observe grooming behavior directly.

Here we present a novel method for automated quantitation of fly grooming behavior based on grayscale video analysis, coupled with a *k*-Nearest Neighbors algorithm (*k*-NN) classifier. The system is easily set up, tracks multiple flies with a single camera, allows the flies to move freely in one dimension, and produces data on a comparatively long time scale. We further show that our system detects grooming behavior with high accuracy when compared to detecting grooming behavior by eye. Using our novel method, we investigate whether *Drosophila*, like mammals, exhibit sickness-associated reduction in grooming behavior. Here we present the first evidence that infected flies show reduced grooming behavior when infected with a bacterial pathogen.

Results

A novel method for grooming quantitation in multiple individuals with continuous and simultaneous sampling.

In order to quantify grooming in multiple flies with continuous sampling, we loaded each of 20 flies into individual tubes that limited their locomotion to approximately one dimension. Tubes were placed within a humidity- and temperature-controlled apparatus, where a single video camera continuously recorded the flies' behavior (Figure 1A). For each video frame of each fly, a binary "fly" matrix was created by comparing each pixel's grayscale values between that frame and a background reference image of the empty apparatus. Threshold cutoff values were used to eliminate various kinds of noise resulting from the apparatus (See Appendix 2 for details).

Using the "fly" matrix, the position of the fly's center in each frame was calculated. The fly's image was also divided into "core" and "periphery" areas using grayscale values (Figure 1B). This allowed us to

quantify grooming because during stereotypical grooming movements, the flies move their periphery without moving their core. In contrast, during locomotion (walking) the flies move both their periphery and core, and during resting, neither the core nor the periphery is displaced.

We examined several quantitative features of the fly's movement from frame to frame. Using Principal Component Analysis (PCA), we chose three components to plot our data points: centroid displacement (DS), difference in core (CC), and difference in periphery (CP). These three features were the most predictive of the fly's behavior from frame to frame. We then applied a *k*-NN algorithm to classify fly behavior for every single frame in each video. This method plots each unclassified data point in three dimensions, using (DS, CC, CP) as (x, y, z) values. The data points in the training set, whose classifications were determined by eye (see Methods) are also plotted.

We adjusted our *k*-NN classifier's constant to 10 for optimal accuracy and efficiency, and automatically classified each fly's behavior in each frame as walking, grooming, or resting. Thus a frame was considered to be a target frame if its 10 nearest neighbors showed DS, CC, and CP values characteristic of grooming behavior. We defined a grooming event as any interval longer than three seconds wherein two conditions were satisfied: first, the majority of frames are target frames, and second, the fly's average speed is much lower than walking speed.

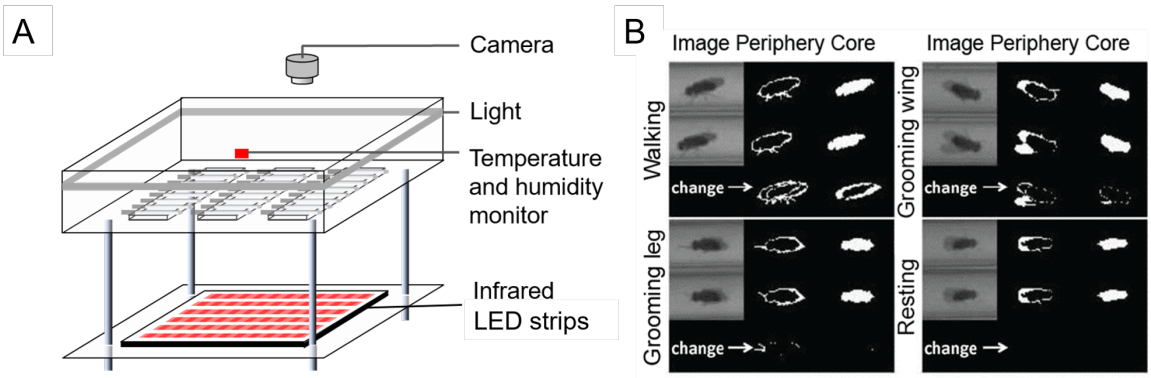

Figure 1. Experimental setup for automated grooming detection and sample data. (A) Schematic of grooming apparatus and recording system. Flies are constrained in individual tubes, continuously illuminated by infrared light from below, and recorded by a digital camera. LED lights on sides of chamber are used to simulate day-night conditions. Temperature and humidity probes are placed in the chamber and connected to the computer. (B) Original and processed images of a fly displaying four behaviors: walking, wing grooming, front leg grooming, and resting. For each mode, we show images of one fly in two neighboring frames, followed by its periphery and core shape. Changes of periphery and core are shown in last row. For a walking fly, changes of periphery and core are at the same level. For grooming fly, the change of periphery pixels is more dramatic than the change of core pixels. For a resting fly, no change in core or periphery pixels occurs.

The automated system accurately measures grooming.

We set out to test the accuracy of our automated system for grooming detection. To test this, we quantified the rate of false negatives and false positives by comparing video footage analyzed by eye with video footage analyzed by the automated program. False negatives result from a failure of the system to detect what was actually grooming behavior, while false positives result from detection of grooming when the fly is actually performing a different behavior.

To measure our automated system's rate of false negatives, we randomly sampled 20 video clips for a total of 460 minutes. Our program successfully detected 95.5% of the grooming detected by eye (Figure 2A). To find our automated system's rate of false positives, we randomly sampled 10% of the total grooming identified by our program visually. We found that 92.1% of grooming time identified by the program was verifiable by eye (Figure 2B).

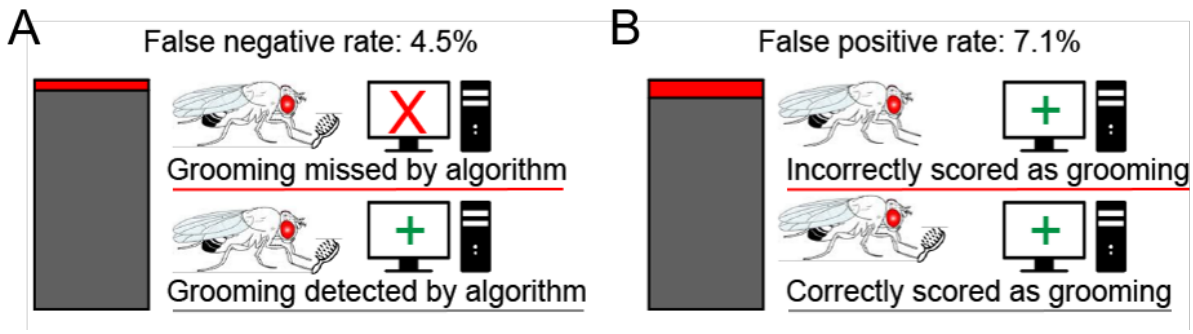

Figure 2. Video was separately classified according to both the automated method and visual observation. The proportion of false negative and positive results yielded by the automated method compared to by eye was quantified. (A) 95.5% of total grooming was detected by the algorithm, while 4.5% was missed. (B) Out of the grooming detected by the algorithm, 92.1% was correctly identified as grooming, while 7.9% was erroneously identified as grooming.

Infected flies show reduced grooming behavior.

Reduced grooming is a well-documented sickness behavior in animals suffering from infection [203]. Therefore, we asked whether sickness behavior in flies is associated with decreased grooming. We injected flies with either sterile media or a liquid culture of the facultative intracellular pathogen, *Listeria monocytogenes*. We found that infected flies groomed ~15% less than un-infected flies (Figure 3A). This reduction was not linked to an overall reduction in distance moved (Figure 3B) or speed of movement (Figure 3C), suggesting that grooming is not simply one of many locomotor activities reduced in infected flies. These results suggest that grooming activity, but not total movement, is reduced in infected flies.

Discussion

Our group has developed a novel, highly accurate method to measure the grooming behavior of multiple flies over multiple days, with high temporal resolution. When verified by eye, the rate of false positives was about 8%, and the rate of false negatives was about 4.5%. One way to reduce error might be to incorporate additional components describing the fly's movement in the analysis. However, we tested many combinations and we found that using these three (CC, CP, DS) was the best choice to balance accuracy and efficiency of the analysis.

By design, our algorithm efficiently classifies behavior when the fly's movement is approximately limited to one dimension. However, flies outside of the laboratory would be expected to instinctively avoid

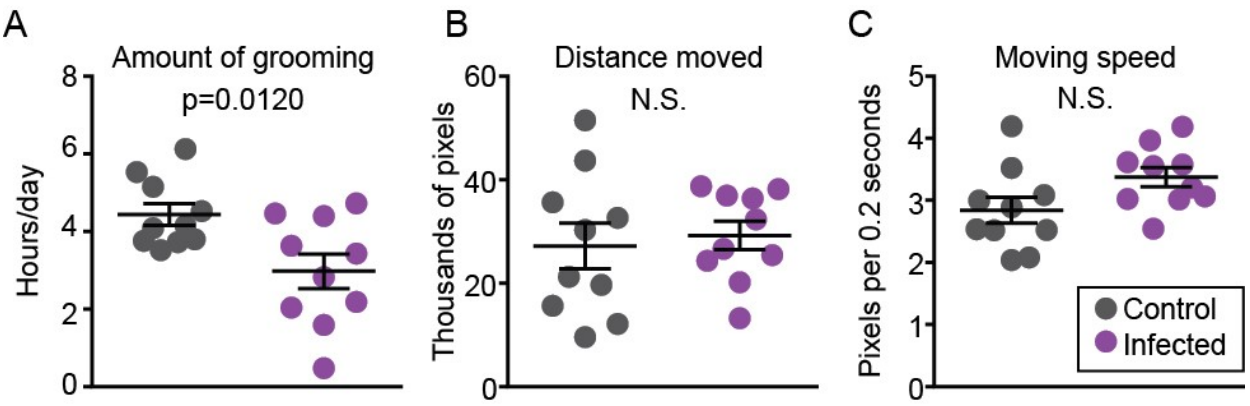

Figure 3. Infected flies show reduced grooming behavior. (A) Time spent grooming was quantified in infected flies (purple) and media-injected controls (gray, p=0.0120). Distance moved (B) and moving speed were (C) also assayed in infected flies and media-injected controls. n=10 for infected flies and controls. Data is represented as mean ± S.E.M.; N.S. denotes p>0.05.

such confinement. Future work should determine whether being confined to one-dimensional movement causes stress or otherwise affects behavioral patterns. This could be accomplished by visual analysis comparing grooming, walking, resting, and flying behavior frequencies for flies in various environments that allow one-, two-, or three-dimensional movement. The utility of this paradigm for investigating behavior will be reinforced if it can be established that one-dimensional confinement has no effect on grooming frequency.

Notably, we found that infected flies showed reduced grooming behavior compared to flies injected with a sterile solution. This suggests that reduced grooming may be a conserved sickness behavior between mammals and invertebrates. However, unlike the lethargic sickness behavior observed in humans, infected flies still had similar or higher (trending) rates of locomotion. Unless confinement to one-dimensional movement, as previously discussed, somehow overrides lethargic sickness behavior, lethargy may not be a conserved sickness behavior in *Drosophila*. Alternatively, future studies may clarify that the profile of sickness behaviors induced depends on the specific pathogen.

A major question in the field of sickness behavior is whether these behaviors are adaptive, and contribute to the animal's survival, or whether they are maladaptive, and are a consequence of or a contributor to pathogenesis. The novel method for quantitation introduced here, combined with the discovery of reduced grooming after infection, will allow us to address this important question about sickness behavior. By screening available *Drosophila* genetic libraries, researchers should be able to

identify mutants that do not respond to infection with reduced grooming. Their infection survival phenotypes may help us learn whether or not reduced grooming is an adaptive sickness behavior.

Methods

Fly strains and food

Wild-type flies used in validation experiments were of strain 2U, while wild-type flies used in infection experiments were Oregon R. Flies were bred and raised on standard food containing cornmeal, molasses, and yeast, with a total concentration of 5-10% sugar and 2-3% yeast. The mixture was boiled for 25 minutes, then let cool for 45 minutes, or until the temperature reached 65°C or less. Methylparaben (Tegosept, 50 g in 300 mL ethanol) and 180 mL of propionic acid were then added as antimicrobial agents. Flies were raised at 25°C, 55–65% humidity on standard food and entrained to a 12h:12h light:dark cycle in a Darwin Chambers incubator for at least three days prior to infection. 5-6 day-old males were used for validation experiments; 5-8 day-old males were injected to be used for infection experiments.

Device and video format

The apparatus was constructed as shown in Figure 1 (a), modeled after the set-up of a typical digital video tracking system (High-Resolution Positionl Program). It contained 20 Pyrex tubes (Trikinetics, PGT5x65) each housing a single fly. The tubes are arranged in two parallel rows of 10. Videos were recorded at the speed of 10 frames per second. The frame size was 1280×960, and grayscale values were calculated on a scale of 0 (black) to 255 (white).

Analysis

The analysis contains several steps: Subtracting background, extracting the fly's image and position, distinguishing between periphery and core, entraining the classifier, and finally, classifiying behavior. The precise equations and constants used for these processes can be found in Appendix 2.

Bacterial cultures

L. monocytogenes was grown overnight at 37°C in 5 mL standing Brain Heart Infusion (Teknova) and diluted to an OD_{600} of 0.10 for injection into flies.

Injections

Infections were performed as described [204] with *Listeria monocytogenes*, strain 10403s. Injections were performed with age-matched, mated males, in vials containing approximately 20 flies. For injections, flies were lightly anesthetized with CO_2. Injections were carried out with a 10 µL Drummond Scientific glass capillary needle (#3-000-210- G), machine-pulled by a Sutter Instrument Co. machine (Model P-30). A custom-modified Tritech microinjector was used to inject 50 nL of liquid into each fly. Volume was calibrated by measuring the diameter of an expelled drop in halocarbon oil 700 (Sigma, H8898) under a layer of mineral oil (Sigma, M8410). All injections were performed between (Zeitgeber time) ZT 5 and ZT 8 to minimize variability from circadian effects on immunity. After injection with *L. monocytogenes*, flies were shipped from New York, NY, to Coral Gables, FL, overnight by FedEx. Infected and un-infected flies were monitored by video for 48 hours.

AUTHOR CONTRIBUTIONS

BQ and SS designed the experimental apparatus. MSH, VWA, BQ, and SS designed experiments. Experiments were performed by BQ (video recording, analysis, and validation) and VWA (infection). BQ, SS, VWA, and MSH produced the manuscript.

V. Conclusion

The research presented in this thesis extends the useful body of knowledge gained by studying immunity using *Drosophila* as a model system. In Chapter II, I demonstrated that circadian-regulated feeding behavior contributes to tolerance of bacterial infection and identifed TORC2 as a novel mediator of tolerance. In Chapter III, I showed that age increases flies' mortality from bacterial infection and reduces their ability to phagocytose bacteria. Finally, in Chapter IV, I presented data indicating that like mammals, *Drosophila* show reduced grooming as a component of sickness behavior. In this section I will attempt to contextualize these findings in terms of our current knowledge and their implications for human health and disease.

Circadian-Regulated Feeding Behavior, Diet, and Immunity

By examining a circadian mutant with increased infection tolerance of bacterial infection, we identified feeding as a circadian-regulated behavior contributing to increased tolerance. Increased tolerance did not depend on metabolic reserves, but rather on acute exposure to two specific nutrients, glucose and amino acids.

Two sets of experiments looking into specific types of amino acids and the method of administration required for tolerance were not included in the final publication and would constitute areas for future investigation. For example, we didn't include a number of experiments we performed attempting to identify an amino acid or subset of amino acids sufficient for tolerance. We attempted to add back or remove random pools of amino acids or single amino acids (but not all of them) from the flies' diets. We obtained many "necessary, but not sufficient" results, and one future direction of research would be to finish these studies in a more comprehensive fashion. In particular, casting the amino acids into random pools was probably not the most elegant approach. We could have classified them according to essential and non-essential amino acids, branched and non-branched chain amino acids (some of which are known to have specific effects on immune physiology [205]), and those amino acids which can be interconverted through biochemical pathways *in vivo*.

Furthermore, while the publication included experiments showing that ingested but not injected

amino acids could induce tolerance, we did not include experiments showing that ingested amino acids might depend on the *Drosophila* microbiota to induce tolerance. These results, while consistent at one time, proved to be inconsistent and variable when repeated at a later date. The reason for this remains unknown but we hypothesize that it may be due to seasonal or other variation in microbiota components. Recent research has indicated that microbiota composition in *Drosophila* is widely variable [206, 207]. The effects of microbiota on amino acid-stimulated tolerance would be a second future direction of interest.

To explore the effects of dietary amino acids on survival of infection, we investigated the role of TORC1 signaling, a canonical amino acid sensing pathway. We found that TORC1 kinase activity oscillates with the circadian cycle. We also uncovered a role for TORC1 in resistance against infection in *Drosophila*. This result was surprising, given that immunosuppressive effects through the TOR pathway in vertebrates are mostly thought to result from inhibition of dendritic cells and T-cells [109], adaptive immune cell types with no clear functional analogs in *Drosophila*. Our data now suggest a role for TORC1 in innate immunity against infection, and potentially open the genetically tractable system of *Drosophila* to investigating TORC1 interactions with innate immune components.

An interesting future direction would be to clarify how TORC1 activity increases resistance. One panel of experiments could use tissue-specific drivers to investigate which tissues TORC1 is required for resistance to infection. Identifying where TORC1 activation is required for resistance may suggest a mechanism. It would also be interesting to determine whether TORC1 induces resistance to other bacteria besides *B. cepacia*. If TORC1 increases resistance to specific pathogens, this may suggest a mechanism for induction of the TORC1 response. Investigating whether TORC1 is involved in resistance to fungal pathogens and viral pathogens could also clarify how TORC1 increases resistance.

One possibility is that TORC1 contributes to resistance through its role in autophagy. Autophagy is important in innate immunity for its role in eliminating intracellular pathogens [208]. Future experiments could use genetic or pharmacological means to specifically inhibit autophagy, and compare whether wild-type flies still showed a survival advantage over flies over-expressing *Tsc1* and *Tsc2*. If wild-type flies still show a survival advantage when autophagy is inhibited, this would suggest that TORC1 affects resistance through a different mechanism. However, if wild-type flies no longer show a survival advantage

when autophagy is inhibited, this would suggest that autophagy may underlie the resistance benefit conferred by TORC1.

In Chapter II, our data also showed a novel role for the less well-known TOR complex 2 as a potent inhibitor of immunity. Loss of TORC2-specific components Rictor or Sin1 caused dramatic increases in survival time after infection and impacted both resistance and tolerance (Figure 7). Our results suggest that TORC1 and TORC2 act in opposition during immunity and we speculate that these complexes may be oppositely circadian-regulated–that is, per^{01} mutants have high TORC1 and low TORC2 activity. It's also possible that per^{01} mutants have altered subcellular localization of the TORC2 complex and that this is what underlies the mutants' increased tolerance. The TORC2 complex must be localized to the plasma membrane to accomplish certain functions [209]. Imaging mutants and controls could determine whether TORC2 is localized to the plasma membrane in per^{01} mutants.

Chapter II also showed that TORC2 inhibition increases survival of infection in *Drosophila*. Our finding was surprising since most immune effects of TOR are thought to act through TORC1. A recent study indicated that Rictor, a component of TORC2, inhibits Toll-like receptor-stimulated cytokine expression in mouse tissue [189]. Thus Sin1, Rictor, and TORC2 may have conserved immune-suppressive effects in both vertebrates and invertebrates. While the direct targets of TORC2 relevant for infection resistance and tolerance remain unknown, their identification will be an important goal of future studies.

To this end, we have few clues as to how reducing TORC2 signaling improves survival of infection. TORC2 is known to be important for actin polymerization and endocytosis in yeast [210]. At least three pieces of evidence suggest why inhibiting actin polymerization might be beneficial to resistance. First, actin polymerization is necessary for enteropathogenic *E. coli* to colonize the gut [211]. Second, *S. typhimurium* depends on actin polymerization to colonize non-phagocytic cells and endocytosis might be detrimental to immunity [211]. Third, many bacteria that replicate in the cytosol are dependent on actin polymerization for mobility [211]. It is less clear how inhibiting actin polymerization might contribute to tolerance or how inhibiting endocytosis might improve survival of infection.

A fourth possible future direction would include identifying the spatio-temporal context in which TORC2 acts to inhibit tolerance, using UAS-RNAi constructs against *rictor* or *Sin1* in combination with

Gal4 drivers. First, it would be prudent to use inducible ubiquitous drivers of RNAi against *rictor* or *Sin1* to verify that the effects of TORC2 inhibition on immunity do not result from developmental abnormalities. Second, tissue-specific drivers could be used to help pinpoint how TORC2 affects immunity. If knockdown of TORC2 function in specific tissues is sufficient to increase survival of infection, this would suggest TORC2 acts to inhibit immunity in these tissues.

Additionally, one could seek to identify interacting components by performing an RNAi-based suppressor screen in *rictor* or *Sin1* mutants, beginning with a small library of previously identified downstream molecular candidates and looking for suppression of increased survival of infection. Such a screen may be complicated by the fact that many of these downstream molecular targets are general cytoskeletal components. Therefore, these experiments should follow those needed to determine the tissue(s) of relevance and the relevant temporal phase.

The cellular and molecular mechanisms that promote host tolerance of infection are not well-understood [150, 212]. *B. cepacia* is a significant opportunistic bacterial pathogen, particularly in hospital settings with susceptible patients [159]. This hospital-acquired infection can be associated with high rates of mortality, up to 50% for severe strains, and is often antibiotic-resistant [190]. Understanding the tolerance mechanisms stimulated by acute glucose and dietary amino acids will help to identify targets for pharmacological treatments. In Chapter II, we identified TORC2 as a potential pharmacological target to increase host survival time after infection, as TORC2 mutants are able to survive infection up to 59% longer than wild type. The potential therapeutic value of TORC2 inhibition has not been explored, as there are currently no known small molecule inhibitors specific to TORC2 and not TORC1. The *Drosophila* model of infection described here may therefore prove useful in screening for such TORC2-specific inhibitors and for further dissection of acute, nutrient-stimulated, TOR-mediated host defenses against bacterial infections such as *B. cepacia*.

Therefore, the final and most exciting future direction suggested by this work is the identification of potential new therapies for systemic bacterial infections. A sensible approach might be to combine both medium- and low-throughput screens. In the first arm of the study, a medium-throughput tissue-culture cell-based screen would test libraries of pharmacological compounds to identify TORC2-specific inhibitors, possibly using a luciferase or other fluorescence based-assays. Since TORC1 signaling

induces translation [213], quantifying the rate of translation in the same samples by alternative fluorescence methods could help determine whether the compound also reduces translation. This would help to eliminate compounds that inhibit both TORC1 and TORC2 signaling. Further validation would then be necessary to show that the candidate TORC2 inhibitors increase tolerance to infection.

To that end, a low-throughput secondary screen in *Drosophila* could be used to determine whether the compounds identified as TORC2 inhibitors in cell culture actually increase immunity to bacterial infection. The compounds could be fed or injected into flies at a variety of dosages. The flies would then be infected with *B. cepacia*; groups with significantly longer survival times would be flagged for follow-up studies. More extensive validation would confirm that the TORC2-specific inhibitors administered to these groups actually increase survival of infection. Bacterial load assays in *Drosophila* could then be used to determine whether these TORC2 inhibitors induce tolerance of or resistance to infection. Mammalian studies could then be used to determine whether the compounds may have clinical and translational relevance to patients, especially sepsis patients.

Aging and Immunity

By comparing young and old flies infected with different types of pathogenic bacteria, we established that older flies become more sensitive to specific infections. Reduced survival of infection by aged flies was associated with increased bacterial load during *S. pneumoniae* infection, but not *L. monocytogenes* infection, suggesting that age reduces both tolerance and resistance. A specific immune response, phagocytosis, was reduced in old flies depending on the time of day. These data suggests that like elderly people, elderly flies undergo functional senescence of the innate immune system. Our results are consistent with the hypothesis that specific immune defense mechanisms become dysregulated with age.

The finding of reduced phagocytosis in old flies presented in Chapter III is consistent with other publications indicating that old flies have reduced phagocytic turnover [92, 93]. Surprisingly, the first of these studies [92] concluded that the proportion of phagocytically active cells declines with age in both males and females, while the second [93] showed no significant difference between old and young virgin females in proportion of active phagocytic cells. The discrepancy may be due to an effect of mating on

immunity, which would not be unprecedented [214, 215]. My experimental setup did not allow me to determine the proportion of phagocytically active cells, but I did observe a lower number of total phagocytic events during the nighttime in old flies compared to young flies. The first group noted an apparent difference in phagocytic activity depending on the time of day, but did not directly test this with a controlled assay [92]. Although I did not directly compare between circadian time points, the age-related difference in phagocytosis was observed only during the second half of the circadian cycle.

Interestingly, the second group found more engulfed bacteria per active phagocyte in older flies. Follow-up experiments clarified that the early steps of phagocytic uptake were unaffected by age, unlike the later steps in phagocytosis, which were slowed in old phagocytes. Thus old phagocytes did not complete phagocytosis by destroying the bacteria, causing an accumulation of phagocytosed bacteria in comparison to young flies. Their data suggested that accumulation of phagosomes in older individuals due to a lack of final degradation in the lysosome.

My protocol did not allow me to quantify the proportion of phagocytically active cells. However, in contrast to in that study, I did not observe an accumulation of phagocytosed bacteria in older individuals. There are several differences in protocol that could account for the difference in our results. First, I used male flies as opposed to used virgin females. Second, our experiments were different with regard to circadian time, phagocytosis time, and sample-processing time. Further studies should be undertaken to clarify the effects of aging on phagocytosis and the circadian regulation of phagocytosis in both sexes of mated and un-mated flies.

Together with the two previous studies on senescence of the phagocytic response in *Drosophila*, the data presented in Chapter III suggests that future research should focus on determining the molecular mechanisms by which phagocytosis is reduced in old flies. As previously stated, I observed reduced phagocytosis in old flies during the dark part of the circadian cycle, but not during the light part of the cycle. We hypothesize that some molecular factor promotes phagocytosis at night and that the induction of this factor is dampened as flies age and lose robust circadian regulation. Consistent with this hypothesis, phagocytosis is known to be circadian-regulated [146].

Two RNAi screens have investigated gene products necessary for phagocytosis of different microbes in *Drosophila* [216, 217]. Other researchers have undertaken microarrays to identify transcripts

differently expressed with age [218, 219] or throughout the circadian cycle [145, 220-222]. Comparing these data sets should provide candidate genes for further research into the underlying molecular mechanisms for the reduction of phagocytosis with age. Alternatively, the factor promoting phagocytosis in young flies at night may be regulated translationally rather than transcriptionally. To this end, using a phagocyte-specific driver with the recently developed ribotag system may allow us to identify specific changes in the proteins translated throughout the circadian cycle and aging. The ability to rescue age-related decline in phagocytosis in *Drosophila* will be an important step towards understanding age-related, immune, and circadian physiologies in humans.

In Chapter III, I showed that old flies are susceptible to some pathogens but not others. Old flies died more quickly after infection by two different "slow-killing" bacteria, but not the "quick-killing" bacterium *P. aeruginosa*. These results are consistent with the hypothesis that old flies are more susceptible to long-lasting infections, but not to more rapid infections. It is worth noting that infecting old flies with *B. cepacia*, bacteria that kill more slowly than *P. aeruginosa*, but faster than the other two pathogens, produced inconsistent results. While these experiments were therefore not included in Chapter III, it is tempting to imagine that *B. cepacia* occupies a unique position on a spectrum of infection kinetics, right on the cusp of where age starts to reduce immunity.

Alternatively, age-related survival phenotypes may exist only with some infections but not others due to a different component of their pathogenesis, such as toxins secreted or bacterial localization within the host. To distinguish between these two possibilities, future experiments should test higher doses of "slow-kill" bacteria, lower doses of "fast-kill" bacteria, and other *Drosophila* models of bacterial infection.

Another possibility is that specific types of bacteria cause age-related survival phenotypes. Intriguingly, old flies in our experiments showed increased sensitivity to infection by the gram-positive pathogens, *S. pneumoniae* and *L. monocytogenes*, but not to the infection by the gram-negative pathogen, *P. aeruginosa.* Testing additional gram-positive and gram-negative bacterial infections would clarify this potential pattern. The results would indicate whether functional immunity to gram-positive pathogens declines with age while immunity to gram-negative pathogens remains intact. Some additional areas of bacterial physiology which may affect age-related infection phenotypes in *Drosophila* are listed in Table 1; others include the ability to form spores or biofilms. Understanding the characteristics that

subject a bacterial infection to age-related changes in functional immunity might help clarify the specific aspects of immunity that change with age.

Bacterium	***L. monocytogenes***	***S. pneumoniae***	***P. aeruginosa***
Age reduces survival?	Yes	Yes	No
Gram stain	+	+	-
Shape	Rod-shaped	Coccoid	Rod-shaped
Oxygen requirement	Facultative anaerobe	Facultative anaerobe	Aerobic, sometimes facultatively anaerobic
Catalase	+	-	+
Oxidase	-	-	+
Lysis method	β-hemolysin	α-hemolysin	ExoU (enzyme)
MDR*?	No	Some	Yes
Phylum	Firmicute	Firmicute	Proteobacteria

Table 1. Comparative characterization of bacteria used for functional immunosenescence assays.
*MDR = Multi Drug Resistant.

Another interesting result shown in Chapter III is that old flies exhibit both reduced tolerance and reduced resistance to bacterial infection. Since phagocytosis is a resistance mechanism, reduced phagocytosis may be responsible for reduced resistance. However, tolerance isn't well understood. With so many known age-related pathologies, one could imagine many mechanisms for old flies' reduced tolerance. Chapter II [142] showed that feeding and diet can affect tolerance of infection. It is known that many metabolic pathologies are associated with aging [58, 223]. Further investigations should focus on the relationship between aging, metabolism, and tolerance.

Tolerance is technically difficult to study because it is functionally defined as longer survival without reduced bacterial load. Therefore any resistance survival phenotype (change in bacterial load) will

mask a tolerance survival phenotype (no change in bacterial load). A possible future direction for both Chapters II and III would be to develop assays for specific mechanisms of infection tolerance that are independent of bacterial load. These might include a Western blot-based assay for TORC2 inhibition, or infection protocols designed to avoid potentially masking resistance effects. In Chapter II, for example, we showed that *Sin1* mutants showed improved tolerance when amino acids were excluded from their diet, but improved resistance when amino acids were included. A final future direction for this work might also include examining how age affects *Sin1* mutants' immune phenotypes, i.e., whether aging impacts tolerance due to inhibition of TORC2.

In summary, the research in Chapter III extends the use of *Drosophila* as a model system for studying immunosenescence. As average lifespan continues to extend, there is significant interest in improving healthspan. The prevalence of infection in the elderly underscores the importance of understanding how aging decreases immunity and developing therapeutic interventions. The fruit fly's short lifespan and conserved innate immune pathways make it an attractive model system for this work.

Reduced grooming as an aspect of sickness behavior

By using a new automated, video-based behavioral assay to examine flies infected with *L. monocytogenes*, we identified reduced grooming as a component of sickness behavior that is conserved in *Drosophila*. Reduced grooming by infected flies was not associated with reduced locomotor activity, suggesting that infection results in a decrease in this specific behavior rather than decreased locomotion generally. Our data suggests that this new method of automated video analysis can be used to accurately quantify grooming behavior and ask questions of biological relevance.

First, the method introduced in Chapter IV represents a significant improvement in methodology for studying grooming behavior. Previously published methods were labor-intensive and lacked the capacity for large sample sizes or long time courses. Our finding that grooming behavior is reduced upon infection proves that this new method is able to generate statistically appropriate data sets. This new method allows us to quantify differences in grooming behavior between experimental groups efficiently, for several days, with temporal precision. As there is currently great interest in mapping the neural circuits that control behavior, this new automated assay will likely prove very useful as a screening tool.

Since mammals exhibit lethargy and depression upon infection [110], I was very surprised that I did not observe depression of locomotor activity in *Drosophila*. Possible reasons include the specific model pathogen selected, the confining nature of the experimental apparatus, and other assay conditions. An alternative way to test whether flies exhibit depressed locomotive behavior after infection is to use a recently developed high-resolution optical method to quantify locomotion [202]. This assay would also allow the experimenter to determine if specific gait parameters were different between infected and uninfected flies' walking movements.

It is possible that lethargy is not a conserved sickness behavior in *Drosophila*. Mammals have a dedicated adaptive immune system for defense against pathogens along with a complex neuroendocrine system. If lethargy in response to sickness is mediated by the adaptive immune system, that would be consistent with its absence in flies. As discussed in Chapter II, metabolism can regulate immune responses. Since temperature regulation can affect metabolism, this is another reason that a specific sickness behavior characteristic of mammals might not be conserved in *Drosophila*.

In contrast to lethargy and depression, I found that sickness behavior in *Drosophila* does include reduced grooming. Previously, it was known that mammals show reduced grooming behavior when infected with different kinds of microbes. My data provides the first evidence that *Drosophila* can also reduce grooming in response to infection. The conservation of this sickness behavior means that we may be able to use this model system to map the molecular signaling cascade that causes reduced grooming as a result of microbial infection. We will also be able to use the automated system to determine whether the reduced grooming response is specific to different types of pathogens or pathogenesis.

An important question in basic biology is whether sickness behavior is adaptive (beneficial to the host) or simply a consequence of debilitation. Using our new automated technology for quantifying grooming behavior, we should be able to identify mutants that do not show reduced grooming behavior after infection. If so, these can be assayed for altered survival of infection to test the benefit of reduced grooming for the host.

It is likely that the canonical immune signaling pathways, Toll and Imd, play a role in reduced grooming as a sickness behavior in *Drosophila*. To this end, we have begun testing a Toll pathway mutant. Preliminary data indicates that, unlike wild type, this mutant does not display reduced grooming in

response to *L. monocytogenes* infection. Upcoming studies will investigate other immunity pathway mutants, including Imd, in order to establish whether reduced grooming is specifically mediated by the Toll signaling pathway, or whether reduced grooming is more generally reflective of pathogenesis. Because the Toll pathway has known effects on immunity and survival of infection, these mutants are not suitable to test the specific benefit of reduced grooming during infection.

Previous work has already identified specific neuronal components involved in grooming [133, 139]. Using similar techniques, future studies could ascertain whether these components are also involved in reducing grooming in response to infection, or whether these neuronal components are independent. These neuronal components can be tested for effects on specific immune mechanisms and, if they do not directly regulate known mechanisms, could be good candidates to test for any specific benefit of reduced grooming during infection.

Furthermore, a low-throughput candidate screen could be able to identify mutations that ablate the reduced grooming in response to infection, but do not appear to be involved in classic immunity pathways nor in general neural circuitry. We can then examine whether these mutants also show reduced survival to bacterial infection, increased survival of bacterial infection, or whether their survival is pathogen-dependent. Alternatively, one could quantify individual variation in survival of infection and test if this correlates with the onset, duration, or extent of reduced grooming during infection. Any positive correlation would suggest that reduced grooming may provide host benefit and point us in the direction of specific candidate genes involved in that parameter of grooming to directly assay survival differences. Finally, one could attempt to induce grooming in sick flies (for example, by sprinkling with a fine powder to stimulate grooming) and assay for any survival benefit. Thus in the future, this model system may help us to determine whether sickness behavior actually confers some benefit to the host animal.

In conclusion, pathogens and hosts have been locked in an evolutionary battle for ages; immunity is essential for any organisms' fitness and for human health. How does circadian regulation impact immunity? How does metabolism affect immunity? What specific changes occur during immunosenescence? Is sickness behavior helpful to an animal host or is it a consequence of pathology? In my thesis chapters, I contribute to our expanding knowledge of these fields by showing that circadian-regulated feeding behavior and TOR signaling modulate survival of infection, that aging causes declines

in specific innate immune functions, and that grooming is a conserved behavior response to sickness in *Drosophila*.

Works Cited

1. Valanne, S., Wang, J.-H., and Rämet, M. (2011). The Drosophila Toll Signaling Pathway. The Journal of Immunology *186*, 649-656.

2. Grewal, S. (2009). Insulin/TOR signaling in growth and homeostasis: a view from the fly world. The international journal of biochemistry & cell biology *41*, 1006-1010.

3. Allada, R. (2010). Circadian organization of behavior and physiology in Drosophila. Annual Review of Physiology *72*, 605-624.

4. Hoebe, K. (2004). The interface between innate and adaptive immunity. Nature Immunology *5*, 971-974.

5. De Gregorio, E., Spellman, P.T., Tzou, P., Rubin, G.M., and Lemaitre, B. (2002). The Toll and Imd pathways are the major regulators of the immune response in Drosophila. The EMBO Journal *21*, 2568-2579.

6. Tanji, T., Hu, X., Weber, A.N.R., and Ip, Y.T. (2007). Toll and IMD pathways synergistically activate an innate immune response in Drosophila melanogaster. Molecular and Cellular Biology *27*, 4578-4588.

7. Lemaitre, B. (2007). The host defense of Drosophila melanogaster. Annual review of immunology *25*, 697-743.

8. Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. Nature (London) *415*, 389-395.

9. Ganz, T. (2003). The role of antimicrobial peptides in innate immunity. Integrative and comparative biology *43*, 300-304.

10. Wang, G. (2014). Human antimicrobial peptides and proteins. Pharmaceuticals (Basel, Switzerland) *7*, 545-594.

11. Christensen, B.M. (2005). Melanization immune responses in mosquito vectors. Trends in parasitology *21*, 192-199.

12. Cerenius, L. (2004). The prophenoloxidase-activating system in invertebrates. Immunological reviews *198*, 116-126.

13. Eleftherianos, I., and Revenis, C. (2012). Role and importance of phenoloxidase in insect hemostasis.

14. Nappi, A., Poirie, M., and Carton, Y. (2009). The role of melanization and cytotoxic by-products in the cellular immune responses of Drosophila against parasitic wasps.

15. Sugumaran, M. (2002). Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. Pigment cell research *15*, 2-9.

16. Wink, D.A., Hines, H.B., Cheng, R.Y.S., Switzer, C.H., Flores-Santana, W., Vitek, M.P., Ridnour, L.A., and Colton, C.A. (2011). Nitric oxide and redox mechanisms in the immune response. Journal of Leukocyte Biology *89*, 873-891.

17. Ulvila, J. (2011). Drosophila phagocytosis - still many unknowns under the surface. APMIS : acta pathologica, microbiologica et immunologica Scandinavica *119*, 651-662.

18. Yutin, N. (2009). The origins of phagocytosis and eukaryogenesis. Biology direct *4*, 9.

19. Pham, L.N., Dionne, M.S., Shirasu-Hiza, M., and Schneider, D.S. (2007). A specific primed immune response in Drosophila is dependent on phagocytes.

20. Shirasu-Hiza, M.M., Dionne, M.S., Pham, L.N., Ayres, J.S., and Schneider, D.S. (2007). Interactions between circadian rhythm and immunity in Drosophila melanogaster. Current Biology *17*, R353-R355.

21. Emery, P., So WV, Kaneko M, Hall JC, Rosbash M. (1998). CRY, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. Cell (Cambridge) *95*, 669-679.

22. Yildirim, E., Chiu JC, Edery I (2015). Identification of light-sensitive phosphorylation sites on PERIOD that regulate the pace of circadian rhythms in Drosophila. Molecular and cellular biology, MCB.00682-00615.

23. Park, J.H., and Hall, J.C. (1998). Isolation and chronobiological analysis of a neuropeptide pigment-dispersing factor gene in Drosophila melanogaster. Journal of biological rhythms *13*, 219-228.

24. Renn, S.C., Park J.H., Rosbash M., Hall J.C., Taghert P.H. (1999). A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. Cell (Cambridge) *99*, 791-802.

25. Zordan, M.A. (2015). Circadian Clock Dysfunction and Psychiatric Disease: Could Fruit Flies have a Say? Frontiers in neurology *6*, 80.

26. Aton, S.J., Colwell CS, Harmar AJ, Waschek J, Herzog ED. (2005). Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons. Nature neuroscience *8*, 476-483.

27. Rosbash, M. (1989). The molecular biology of circadian rhythms. Neuron (Cambridge, Mass.) *3*, 387-398.

28. Shaw, P.J., Cirelli C, Greenspan RJ, Tononi G. (2000). Correlates of sleep and waking in Drosophila melanogaster. Science (New York, N.Y.) *287*, 1834-1837.

29. Sarov-Blat, L., So WV, Liu L, Rosbash M. (2000). The Drosophila takeout gene is a novel molecular link between circadian rhythms and feeding behavior. Cell (Cambridge) *101*, 647-656.

30. Kaneko, H., Head, Lauren M., Ling, J., Tang, X., Liu, Y., Hardin, Paul E., Emery, P., and Hamada, Fumika N. (2012). Circadian Rhythm of Temperature Preference and Its Neural Control in Drosophila. Current Biology *22*, 1851-1857.

31. Myers, E.M., Yu, J., and Sehgal, A. (2003). Circadian Control of Eclosion: Interaction between a Central and Peripheral Clock in Drosophila melanogaster. Current Biology *13*, 526-533.

32. Duffy, J.F. (2015). Aging and Circadian Rhythms. Sleep medicine clinics *10*, 423-434.

33. Montgomery, R.R. (2015). Paradoxical changes in innate immunity in aging: recent progress and new directions. Journal of leukocyte biology *98*, 937-943.

34. Ortman, J.M., Velkoff, Victoria A., Hogan, Howard (2014). An Aging Nation: The Older Population in the United States. Population Estimates and Projections.

35. Jacobzone S, O.H. (2002). Ageing and Health Care Costs. In Internationale Politik und Gesellschaft Online (International Politics and Society).

36. Opal, S.M. (2005). The immunopathogenesis of sepsis in elderly patients. Clinical infectious diseases *41 Suppl 7*, S504-S512.

37. McCay, C.M., Crowell, M.F., and Maynard, L.A. (1935). The effect of retarded growth upon the length of life span and upon the ultimate body size. 1935.

38. Heilbronn, L.K. (2003). Calorie restriction and aging: review of the literature and implications for studies in humans. The American journal of clinical nutrition *78*, 361-369.

39. Kenyon, C.J. (2010). The genetics of ageing. Nature (London) *464*, 504-512.

40. Lee, C. (2016). Dietary restriction with and without caloric restriction for healthy aging. F1000 research *5*.

41. Seals, D.R. (2014). Translational geroscience: emphasizing function to achieve optimal longevity. Aging (Albany, NY.) *6*, 718-730.

42. Bitterman, K.J., Medvedik, O., and Sinclair, D.A. (2003). Longevity regulation in Saccharomyces cerevisiae: linking metabolism, genome stability, and heterochromatin.

43. Hayflick, L. (1961). The serial cultivation of human diploid cell strains. Experimental cell research *25*, 585-621.

44. Vijg, J. (2013). Genome instability and aging. Annual review of physiology *75*, 645-668.

45. Bratic, A. (2013). The role of mitochondria in aging. The Journal of clinical investigation *123*, 951-957.

46. Fang, E.F. (2016). Nuclear DNA damage signalling to mitochondria in ageing. Nature reviews. Molecular cell biology.

47. Gladyshev, V.N. (2014). The free radical theory of aging is dead. Long live the damage theory! Antioxidants & redox signaling *20*, 727-731.

48. Serrano, M. (2007). Cancer and ageing: convergent and divergent mechanisms. Nature reviews. Molecular cell biology *8*, 715-722.

49. Murphy, M.P. (2009). How mitochondria produce reactive oxygen species. Biochemical journal *417*, 1-13.

50. López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The Hallmarks of Aging. Cell *153*, 1194-1217.

51. Weber, T.A. (2010). Impaired quality control of mitochondria: aging from a new perspective. Experimental gerontology *45*, 503-511.

52. Jensen, M.B. (2014). Mitochondrial proteostasis in the control of aging and longevity. Cell metabolism *20*, 214-225.

53. Salminen, A. (2014). Krebs cycle dysfunction shapes epigenetic landscape of chromatin: novel insights into mitochondrial regulation of aging process. Cellular signalling *26*, 1598-1603.

54. Quirós, P.M. (2015). New roles for mitochondrial proteases in health, ageing and disease. Nature reviews. Molecular cell biology *16*, 345-359.

55. Berlett, B.S., and Stadtman, E.R. (1997). Protein Oxidation in Aging, Disease, and Oxidative Stress. Journal of Biological Chemistry *272*, 20313-20316.

56. Yun, J. (2014). Mitohormesis. Cell metabolism *19*, 757-766.

57. Calabrese, E.J. (2002). Defining hormesis. Human & experimental toxicology *21*, 91-97.

58. Owusu-Ansah E, S.W., Perrimon N (2013). Muscle mitohormesis promotes longevity via systemic repression of insulin signaling. Cell (Cambridge) *155*, 699-712.

59. Kenyon C, C.J., Gensch E, Rudner A, Tabtiang R. (1993). A C. elegans mutant that lives twice as long as wild type. Nature (London) *366*, 461-464.

60. Kimura KD, T.H., Liu Y, Ruvkun G (1997). daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. Science (New York, N.Y.) *277*, 942-946.

61. Zarse K, S.S., Groth M, Priebe S, Beuster G, Kuhlow D, Guthke R, Platzer M, Kahn CR, Ristow M. (2012). Impaired insulin/IGF1 signaling extends life span by promoting mitochondrial L-proline catabolism to induce a transient ROS signal. Cell metabolism *15*, 451-465.

62. Qiu X, B.K., Hirschey MD, Verdin E, Chen D. (2010). Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. Cell metabolism *12*, 662-667.

63. Jovaisaite, V. (2014). The mitochondrial unfolded protein response, a conserved stress response pathway with implications in health and disease. Journal of experimental biology *217*, 137-143.

64. Schieber, M. (2014). ROS function in redox signaling and oxidative stress. Current biology *24*, R453-R462.

65. Schulz TJ, W.D., Isken F, Voigt A, Laube B, Thierbach R, Kuhlow D, Zarse K, Schomburg L, Pfeiffer AF, Tschöpe C, Ristow M. (2010). Activation of mitochondrial energy metabolism protects against cardiac failure. Aging (Albany, NY.) *2*, 843-853.

66. Finley, L., Carracedo A, Lee J, Souza A, Egia A, Zhang J, Teruya-Feldstein J, Moreira PI, Cardoso SM, Clish CB, Pandolfi PP, Haigis MC (2011). SIRT3 opposes reprogramming of cancer cell metabolism through HIF1α destabilization. Cancer cell *19*, 416-428.

67. Chung, H.Y. (2009). Molecular inflammation: underpinnings of aging and age-related diseases. Ageing research reviews *8*, 18-30.

68. Franceschi, C. (2014). Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. The journals of gerontology. Series A, Biological sciences and medical sciences *69 Suppl 1*, S4-S9.

69. Jackson, M., Neuzil KM, Thompson WW, Shay DK, Yu O, Hanson CA, Jackson LA. (2004). The burden of community-acquired pneumonia in seniors: results of a population-based study. Clinical infectious diseases *39*, 1642-1650.

70. Nasa, P., Juneja, D., and Singh, O. (2012). Severe sepsis and septic shock in the elderly: An overview. World journal of critical care medicine *1*, 23-30.

71. Koh K, E.J., Hendricks JC, Sehgal A. (2006). A Drosophila model for age-associated changes in sleep:wake cycles. Proceedings of the National Academy of Sciences - PNAS *103*, 13843-13847.

72. Laboratory, T.J. (1996). “Baseline lifespan data”.

73. Tatar, M. (2014). Nutrient control of Drosophila longevity. Trends in endocrinology and metabolism *25*, 509-517.

74. Ristow, M. (2011). Extending life span by increasing oxidative stress. Free radical biology & medicine *51*, 327-336.

75. Katewa, S.D. (2011). Role of TOR signaling in aging and related biological processes in Drosophila melanogaster. Experimental gerontology *46*, 382-390.

76. Gomez, C.R. (2008). Innate immunity and aging. Experimental gerontology *43*, 718-728.

77. Panda, A. (2009). Human innate immunosenescence: causes and consequences for immunity in old age. Trends in immunology *30*, 325-333.

78. Shaw, A.C. (2010). Aging of the innate immune system. Current opinion in immunology *22*, 507-513.

79. Vasto, S. (2007). Inflammatory networks in ageing, age-related diseases and longevity. Mechanisms of ageing and development *128*, 83-91.

80. Kline, K.A. (2015). Infection in an aging population. Current opinion in microbiology *29*, 63-67.

81. Shaw, A.C. (2013). Age-dependent dysregulation of innate immunity. Nature reviews. Immunology *13*, 875-887.

82. Ramsden, S., and Cheung YY, S.L. (2008). Functional analysis of the Drosophila immune response during aging. Aging cell *7*, 225-236.

83. Khan, I.P., NG (2013). The aging of the immune response in Drosophila melanogaster. The journals of gerontology. Series A, Biological sciences and medical sciences *68*, 129-135.

84. Felix, T., Hughes KA, Stone EA, Drnevich JM, Leips J. (2012). Age-specific variation in immune response in Drosophila melanogaster has a genetic basis. Genetics (Austin) *191*, 989-1002.

85. Ancoli-Israel, S., and Martin, J. (2006). Insomnia and daytime napping in older adults. Journal of clinical sleep medicine *2*, 333-342.

86. Roenneberg, T. (2007). Epidemiology of the human circadian clock. Sleep medicine reviews *11*, 429-438.

87. Pagani L, S.K., Meier F, Izakovic J, Roemer K, Viola A, Cajochen C, Wirz-Justice A, Brown SA, Eckert A. (2011). Serum factors in older individuals change cellular clock properties. Proceedings of the National Academy of Sciences - PNAS *108*, 7218-7223.

88. Foley, D.J., Monjan AA, Brown SL, Simonsick EM, Wallace RB, Blazer DG. (1995). Sleep complaints among elderly persons: an epidemiologic study of three communities. Sleep (New York, N.Y.) *18*, 425-432.

89. Crowley, K. (2011). Sleep and sleep disorders in older adults. Neuropsychology review *21*, 41-53.

90. Nakamura TJ, N.W., Yamazaki S, Kudo T, Cutler T, Colwell CS, Block GD. (2011). Age-related decline in circadian output. The Journal of neuroscience *31*, 10201-10205.

91. Weinert, D. (2000). Age-dependent changes of the circadian system. Chronobiology international *17*, 261-283.

92. Mackenzie, D.K., Bussière LF, Tinsley MC. (2011). Senescence of the cellular immune response in Drosophila melanogaster. Experimental gerontology *46*, 853-859.

93. Horn, L., Leips J, Starz-Gaiano M. (2014). Phagocytic ability declines with age in adult Drosophila hemocytes. Aging cell *13*, 719-728.

94. Ayres, J.S., and Schneider, D.S. (2009). The Role of Anorexia in Resistance and Tolerance to Infections in Drosophila. PLoS Biology *7*, e1000150.

95. Katona, P., and Katona-Apte, J. (2008). The Interaction between Nutrition and Infection. Clinical Infectious Diseases *46*, 1582-1588.

96. Finfer, S. (2014). Clinical controversies in the management of critically ill patients with severe sepsis: resuscitation fluids and glucose control. Virulence *5*, 200-205.

97. Song F, Z.L., Han L, Xie GH, Xiao C, Zhao B, Hu YQ, Wang SY, Qin CJ, Zhang Y, Lai DM, Cui P, Fang XM. (2014). Intensive insulin therapy for septic patients: a meta-analysis of randomized controlled trials. BioMed research international *2014*, 1-10.

98. Yoneyama S, T.H., Yamaguchi R, Tadano S, Ohkohchi N. (2015). The negative impact of insulin therapy for acute hyperglycemia secondary to glucose load on plasma amino acid profiles in a rat model of sepsis. European surgical research *54*, 34-43.

99. Liao, X.-H., Majithia A, Huang X, Kimmel AR. (2008). Growth control via TOR kinase signaling, an intracellular sensor of amino acid and energy availability, with crosstalk potential to proline metabolism. Amino acids *35*, 761-770.

100. Wang T, B.R., Lao U, Kuo Y, Edgar BA. (2012). LST8 regulates cell growth via target-of-rapamycin complex 2 (TORC2). Molecular and cellular biology *32*, 2203-2213.

101. Hara, K., Maruki Y, Long X, Yoshino K, Oshiro N, Hidayat S, Tokunaga C, Avruch J, Yonezawa K. (2002). Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell (Cambridge) *110*, 177-189.

102. Kim D-H, S.D., Ali SM, King JE, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell (Cambridge) *110*, 163-175.

103. Yang Q, I.K., Ikenoue T, Guan KL. (2006). Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. Genes & development *20*, 2820-2832.

104. Sarbassov D, A.S., Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Current biology *14*, 1296-1302.

105. Kim E, G.-H.P., Li L, Neufeld TP, Guan KL. (2008). Regulation of TORC1 by Rag GTPases in nutrient response. Nature cell biology *10*, 935-945.

106. Wullschleger, S. (2006). TOR signaling in growth and metabolism. Cell (Cambridge) *124*, 471-484.

107. Kimball, S.R., Shantz LM, Horetsky RL, Jefferson LS. (1999). Leucine regulates translation of specific mRNAs in L6 myoblasts through mTOR-mediated changes in availability of eIF4E and phosphorylation of ribosomal protein S6. The Journal of biological chemistry *274*, 11647-11652.

108. Jacinto, E., Loewith R, Schmidt A, Lin S, Rüegg MA, Hall A, Hall MN. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nature cell biology *6*, 1122-1128.

109. Thomson, A.W., Turnquist, H.R., and Raimondi, G. (2009). Immunoregulatory functions of mTOR inhibition. Nat Rev Immunol *9*, 324-337.

110. Hart, B.L. (1988). Biological basis of the behavior of sick animals. Neuroscience and biobehavioral reviews *12*, 123-137.

111. Dantzer, R. (2007). Twenty years of research on cytokine-induced sickness behavior. Brain, behavior, and immunity *21*, 153-160.

112. Silverman, M.N., Mukhopadhyay P, Belyavskaya E, Tonelli LH, Revenis BD, Doran JH, Ballard BE, Tam J, Pacher P, Sternberg EM. (2013). Glucocorticoid receptor

dimerization is required for proper recovery of LPS-induced inflammation, sickness behavior and metabolism in mice. Molecular psychiatry *18*, 1006-1017.

113. MacDonald L, and Hazi A, P.A., Kent S. (2014). Calorie restriction dose-dependently abates lipopolysaccharide-induced fever, sickness behavior, and circulating interleukin-6 while increasing corticosterone. Brain, behavior, and immunity *40*, 18-26.

114. R, B.S.M.T.B.J.A.P.D.N.D.H.P.K.J.L.X.M.L.V.D.L.H.N.N. (2013). Systemic immune activation leads to neuroinflammation and sickness behavior in mice. Mediators of inflammation *2013*, 1-14.

115. Tizard, I. (2008). Sickness behavior, its mechanisms and significance. Animal health research reviews *9*, 87-99.

116. Fogsgaard, K., Røntved CM, Sørensen P, Herskin MS. (2012). Sickness behavior in dairy cows during Escherichia coli mastitis. Journal of dairy science *95*, 630-638.

117. Adamo, S.A. (1998). The specificity of behavioral fever in the cricket Acheta domesticus. The Journal of Parasitology *84*, 529-533.

118. Louis, C., Jourdan M, Cabanac M. (1986). Behavioral fever and therapy in a rickettsia-infected Orthoptera. American journal of physiology. Regulatory, integrative and comparative physiology *250*, R991-995.

119. Bundey, S.R.S.D.P.R.S.D.R.C.A. (2003). Eicosanoid involvement in the regulation of behavioral fever in the desert locust, Schistocerca gregaria. Archives of insect biochemistry and physiology *52*, 183-192.

120. Starks, P.T., Blackie CA, Seeley TD. (2000). Fever in honeybee colonies. Die Naturwissenschaften *87*, 229-231.

121. Adamo, S.A., Fidler TL, Forestell CA. (2007). Illness-induced anorexia and its possible function in the caterpillar, Manduca sexta. Brain, behavior, and immunity *21*, 292-300.

122. Liehl P, B.M., Vodovar N, Boccard F, Lemaitre B. (2006). Prevalence of local immune response against oral infection in a Drosophila/Pseudomonas infection model. PLoS pathogens *2*, e56.

123. Vodovar N, V.M., Liehl P, Basset A, Degrouard J, Spellman P, Boccard F, Lemaitre B. (2005). Drosophila host defense after oral infection by an entomopathogenic Pseudomonas species. Proceedings of the National Academy of Sciences - PNAS *102*, 11414-11419.

124. Dantzer, R. (2009). Cytokine, Sickness Behavior, and Depression. Immunology and allergy clinics of North America *29*, 247-264.

125. Sachs, B.D. (1988). The development of grooming and its expression in adult animals. Annals of the New York Academy of Sciences *525*, 1-17.

126. Müller-Schwarze D, M.-S.C., Singer AG, Silverstein RM. (1974). Mammalian pheromone: identification of active component in the subauricular scent of the male pronghorn. Science (New York, N.Y.) *183*, 860-862.

127. Seyfarth, R.M. (1977). A model of social grooming among adult female monkeys. Journal of theoretical biology *65*, 671-698.

128. Thiessen, D., Graham M, Perkins J, Marcks S. (1977). Temperature regulation and social grooming in the Mongolian gerbil (Meriones unguiculatus). Behavioral biology *19*, 279-288.

129. Patenaude, F., and Bovet, J. (1984). Self-grooming and social grooming in the North American beaver, Castor canadensis. Canadian Journal of Zoology *62*, 1872-1878.

130. Schino, G.A., A (2015). Short-term costs and benefits of grooming in Japanese macaques. Primates *56*, 253-257.

131. Szebenyi, A.L. (1969). Cleaning behaviour in Drosophila melanogaster. Animal Behaviour *17*, 641-651.

132. Böröczky, K., Wada-Katsumata, A., Batchelor, D., Zhukovskaya, M., and Schal, C. (2013). Insects groom their antennae to enhance olfactory acuity. Proceedings of the National Academy of Sciences *110*, 3615-3620.

133. Seeds, A.M., Ravbar, P., Chung, P., Hampel, S., Midgley, F.M., Mensh, B.D., and Simpson, J.H. (2014). A suppression hierarchy among competing motor programs drives sequential grooming in Drosophila. Elife *3*, e02951.

134. Carlin, N.F., and Hölldobler, B. (1986). The kin recognition system of carpenter ants (Camponotus spp.). Behavioral Ecology and Sociobiology *19*, 123-134.

135. Ozaki, M., Wada-Katsumata, A., Fujikawa, K., Iwasaki, M., Yokohari, F., Satoji, Y., Nisimura, T., and Yamaoka, R. (2005). Ant nestmate and non-nestmate discrimination by a chemosensory sensillum. Science *309*, 311-314.

136. Yanagawa A, G.A., Marion-Poll F (2014). Hygienic grooming is induced by contact chemicals in Drosophila melanogaster. Frontiers in behavioral neuroscience *8*, 254.

137. Zhukovskaya, M., Yanagawa, A., and Forschler, B.T. (2013). Grooming behavior as a mechanism of insect disease defense. Insects *4*, 609-630.

138. Kaur, K., Simon AF, Chauhan V, Chauhan A (2015). Effect of bisphenol A on Drosophila melanogaster behavior--a new model for the studies on neurodevelopmental disorders. Behavioural brain research *284*, 77-84.

139. Hampel, S., Franconville, R., Simpson, J.H., and Seeds, A.M. (2015). A neural command circuit for grooming movement control. eLife.

140. Pitmon E, S.G., Parkhurst SJ, Wolf FW, Kehne G, Taylor M, Lebestky T (2016). The D1 family dopamine receptor, DopR, potentiates hind leg grooming behavior in Drosophila. Genes, brain and behavior *15*, 327-334.

141. Bland, N.D.R.P.T.J.S.A.T.A.I.R. (2009). Locomotor and geotactic behavior of Drosophila melanogaster over-expressing neprilysin 2. Peptides (New York, N.Y. : 1980) *30*, 571-574.

142. Allen, V.W., O'Connor RM; Ulgherait M; Zhou CG; Stone EF; Hill VM; Murphy KR; Canman JC; Ja WW; Shirasu-Hiza MM (2016). period-Regulated Feeding Behavior and TOR Signaling Modulate Survival of Infection. Current Biology *26*, 184-194.

143. Lowrey, P.L., and Takahashi, J.S. (2004). Mammalian circadian biology: elucidating genome-wide levels of temporal organization.

144. Hardin, P.E. (2005). The circadian timekeeping system of Drosophila. Current biology *15*, R714-R722.

145. Lin, Y., Han, M., Shimada, B., Wang, L., Gibler, T.M., Amarakone, A., Awad, T.A., Stormo, G.D., Van Gelder, R.N., and Taghert, P.H. (2002). Influence of the period-dependent circadian clock on diurnal, circadian, and aperiodic gene expression in Drosophila melanogaster. Proceedings of the National Academy of Sciences *99*, 9562-9567.

146. Stone, E.F., Fulton, B.O., Ayres, J.S., Pham, L.N., Ziauddin, J., and Shirasu-Hiza, M.M. (2012). The circadian clock protein timeless regulates phagocytosis of bacteria in Drosophila. PLoS Pathog *8*, e1002445.

147. Brennan, C.A. (2004). Drosophila: the genetics of innate immune recognition and response. Annual review of immunology *22*, 457-483.

148. Lemaitre B, N.E., Michaut L, Reichhart JM, Hoffmann JA. (1996). The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell (Cambridge) *86*, 973-983.

149. Schneider, D.S. (2008). Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases. Nature reviews. Immunology *8*, 889-895.

150. Medzhitov, R. (2012). Disease tolerance as a defense strategy. Science (New York, N.Y.) *335*, 936-941.

151. Råberg, L. (2007). Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. Science (New York, N.Y.) *318*, 812-814.

152. Ayres, J.S., N., F., and DS, S. (2008). Identification of Drosophila mutants altering defense of and endurance to Listeria monocytogenes infection. Genetics (Austin) *178*, 1807-1815.

153. Dionne, M., Pham LN, Shirasu-Hiza M, Schneider DS. (2006). Akt and FOXO dysregulation contribute to infection-induced wasting in Drosophila. Current biology *16*, 1977-1985.

154. Chambers, M.C., Song KH, Schneider DS (2012). Listeria monocytogenes infection causes metabolic shifts in Drosophila melanogaster. PloS one *7*, e50679.

155. Brown, A.E.B.J.C.P.L.P. (2009). Short-term starvation of immune deficient Drosophila improves survival to gram-negative bacterial infections. PloS one *4*, e4490.

156. Xu K, Z.X., Sehgal A (2008). Regulation of feeding and metabolism by neuronal and peripheral clocks in Drosophila. Cell metabolism *8*, 289-300.

157. Green, C.B., Takahashi, J.S., and Bass, J. (2008). The Meter of Metabolism. Cell *134*, 728-742.

158. Lee, J.-E.I., I (2008). Circadian regulation in the ability of Drosophila to combat pathogenic infections. Current biology *18*, 195-199.

159. Lipuma, J. (2005). Update on the Burkholderia cepacia complex. Current opinion in pulmonary medicine *11*, 528-533.

160. Bar-Peled, L. (2014). Regulation of mTORC1 by amino acids. Trends in cell biology *24*, 400-406.

161. Ikai, N. (2011). The reverse, but coordinated, roles of Tor2 (TORC1) and Tor1 (TORC2) kinases for growth, cell cycle and separase-mediated mitosis in Schizosaccharomyces pombe. Open biology *1*, 110007-110007.

162. Delgoffe, G.M., Kole TP, Zheng Y, Zarek PE, Matthews KL, Xiao B, Worley PF, Kozma SC, Powell JD. (2009). The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. Immunity (Cambridge, Mass.) *30*, 832-844.

163. Castonguay-Vanier J, V.L., Tremblay J, Déziel E (2010). Drosophila melanogaster as a model host for the Burkholderia cepacia complex. PloS one *5*, e11467.

164. Schneider, D.S., Ayres, J.S., Brandt, S.M., Costa, A., Dionne, M.S., Gordon, M.D., Mabery, E.M., Moule, M.G., Pham, L.N., and Shirasu-Hiza, M.M. (2007). Drosophila eiger mutants are sensitive to extracellular pathogens. PLoS Pathog *3*, e41.

165. Schwager S, A.K., Köthe M, Feldmann F, Givskov M, Carlier A, Eberl L. (2013). Identification of Burkholderia cenocepacia strain H111 virulence factors using nonmammalian infection hosts. Infection and immunity *81*, 143-153.

166. Ayres, J.S., and Schneider, D.S. (2008). A signaling protease required for melanization in Drosophila affects resistance and tolerance of infections. PLoS biology *6*, 2764-2773.

167. Deshpande, S.A. (2014). Quantifying Drosophila food intake: comparative analysis of current methodology. Nature methods *11*, 535-540.

168. Ja, W.W., Carvalho, G.B., Zid, B.M., Mak, E.M., Brummel, T., and Benzer, S. (2009). Water- and nutrient-dependent effects of dietary restriction on Drosophila lifespan. Proceedings of the National Academy of Sciences *106*, 18633-18637.

169. William, W.J., Carvalho, G.B., Mak, E.M., Noelle, N., Fang, A.Y., Liong, J.C., Brummel, T., and Benzer, S. (2007). Prandiology of Drosophila and the CAFE assay. Proceedings of the National Academy of Sciences *104*, 8253-8256.

170. Ikeya, T., Broughton S, Alic N, Grandison R, Partridge L. (2009). The endosymbiont Wolbachia increases insulin/IGF-like signalling in Drosophila. Proceedings of the Royal Society. B, Biological sciences *276*, 3799-3807.

171. Xu J, H.K., Sabin L, Yasunaga A, Subramanian H, Lamborn I, Gordesky-Gold B, Cherry S (2013). ERK signaling couples nutrient status to antiviral defense in the insect gut. Proceedings of the National Academy of Sciences - PNAS *110*, 15025-15030.

172. Hang, S., Purdy AE, Robins WP, Wang Z, Mandal M, Chang S, Mekalanos JJ, Watnick P (2014). The acetate switch of an intestinal pathogen disrupts host insulin signaling and lipid metabolism. Cell host & microbe *16*, 592-604.

173. DiAngelo, J.R., Bland, M.I., Bambina, S., Cherry, S., and Birnbaum, M.J. (2009). The immune response attenuates growth and nutrient storage in Drosophila by reducing insulin signaling. Proceedings of the National Academy of Sciences - PNAS *106*, 20853-20858.

174. Konopka, R.J., and Benzer, S. (1971). Clock mutants of Drosophila melanogaster. Proc Natl Acad Sci U S A *68*, 2112-2116.

175. Loewith R, J.E., Wullschleger S, Lorberg A, Crespo JL, Bonenfant D, Oppliger W, Jenoe P, Hall MN (2002). Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Molecular cell *10*, 457-468.

176. Lee NN, W.C., Nobil AM, Rauktys AE, Messina MP, Dabora SL. (2009). Rapamycin weekly maintenance dosing and the potential efficacy of combination sorafenib plus rapamycin but not atorvastatin or doxycycline in tuberous sclerosis preclinical models. BMC pharmacology *9*, 8.

177. Huang, J.M.B. (2008). The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. Biochemical journal *412*, 179-190.

178. Foldenauer, M.E.B., McClellan SA, Berger EA, Hazlett LD (2013). Mammalian target of rapamycin regulates IL-10 and resistance to Pseudomonas aeruginosa corneal infection. The Journal of immunology (1950) *190*, 5649-5658.

179. Pollizzi, K.N. (2015). Regulation of T cells by mTOR: the known knowns and the known unknowns. Trends in immunology *36*, 13-20.

180. Jacinto, E., Facchinetti V, Liu D, Soto N, Wei S, Jung SY, Huang Q, Qin J, Su B (2006). SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell (Cambridge) *127*, 125-137.

181. Khapre RV, K.A., Patel S, Dubrovsky Y, Wrobel M, Antoch MP, Kondratov RV (2014). Metabolic clock generates nutrient anticipation rhythms in mTOR signaling. Aging (Albany, NY.) *6*, 675-689.

182. Khapre RV, K.A., Patel S, Dubrovsky Y, Wrobel M, Antoch MP, Kondratov RV. (2014). BMAL1-dependent regulation of the mTOR signaling pathway delays aging. Aging (Albany, NY.) *6*, 48-57.

183. Dimitroff, B., Howe K, Watson A, Campion B, Lee HG, Zhao N, O'Connor MB, Neufeld TP, Selleck SB (2012). Diet and energy-sensing inputs affect TorC1-mediated axon misrouting but not TorC2-directed synapse growth in a Drosophila model of tuberous sclerosis. PloS one *7*, e30722.

184. Jevtov, I., Zacharogianni, M., van Oorschot, M.M., van Zadelhoff, G., Aguilera-Gomez, A., Vuillez, I., Braakman, I., Hafen, E., Stocker, H., and Rabouille, C. (2015). TORC2 mediates the heat stress response in Drosophila by promoting the formation of stress granules. Journal of cell science *128*, 2497-2508.

185. Kuo, Y., Huang, H., Cai, T., and Wang, T. (2015). Target of Rapamycin Complex 2 regulates cell growth via Myc in Drosophila. Scientific reports *5*, 10339.

186. Hietakangas, V.C., Stephen (2007). Re-evaluating AKT regulation: role of TOR complex 2 in tissue growth. Genes & development *21*, 632-637.

187. Koike-Kumagai, M., Yasunaga, K., Morikawa, R., Kanamori, T., and Emoto, K. (2009). The target of rapamycin complex 2 controls dendritic tiling of Drosophila sensory

neurons through the Tricornered kinase signalling pathway. The EMBO journal *28*, 3879-3892.

188. Natarajan, R.T.-V., D.; Wairkar, Y.P. (2013). Tuberous sclerosis complex regulates Drosophila neuromuscular junction growth via the TORC2/Akt pathway. Human molecular genetics *22*, 2010-2023.

189. Brown, J.W.H.S.J.G.D.M.M. (2011). Mammalian target of rapamycin complex 2 (mTORC2) negatively regulates Toll-like receptor 4-mediated inflammatory response via FoxO1. The Journal of biological chemistry *286*, 44295-44305.

190. Woods CW, B.A., LiPuma JJ, Alexander BD, Clements DA, Weber DJ, Moore CM, Reller LB, Kaye KS (2004). Virulence associated with outbreak-related strains of Burkholderia cepacia complex among a cohort of patients with bacteremia. Clinical infectious diseases *38*, 1243-1250.

191. Spencer, R.C. (1995). The emergence of epidemic, multiple-antibiotic-resistant Stenotrophomonas (Xanthomonas) maltophilia and Burkholderia (Pseudomonas) cepacia. The Journal of hospital infection *30 Suppl*, 453-464.

192. Krishnan NN, D.A., Giebultowicz JM. (2008). Circadian regulation of response to oxidative stress in Drosophila melanogaster. Biochemical and biophysical research communications *374*, 299-303.

193. Tapon N, I.N., Dickson BJ, Treisman JE, Hariharan IK (2001). The Drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. Cell (Cambridge) *105*, 345-355.

194. McGowan MW, A.J., Strandbergh DR, Zak B (1983). A peroxidase-coupled method for the colorimetric determination of serum triglycerides. Clinical chemistry (Baltimore, Md.) *29*, 538-542.

195. Ja W. W., C.G.B., Mak E.M., de la Rosa N.N., Fang A.Y., Liong J.C., Brummel T., Benzer S. (2007). Prandiology of Drosophila and the CAFE assay. Proceedings of the National Academy of Sciences - PNAS *104*, 8253-8256.

196. Horn, L., Leips, J., and Starz-Gaiano, M. (2014). Phagocytic ability declines with age in adult Drosophila hemocytes. Aging Cell *13*, 719-728.

197. Rakshit, K., Krishnan, N., Guzik, E.M., Pyza, E., and Giebultowicz, J.M. (2012). Effects of aging on the molecular circadian oscillations in Drosophila.

198. Rakshit, K., and Giebultowicz, J.M. (2013). Cryptochrome restores dampened circadian rhythms and promotes healthspan in aging Drosophila.

199. Mansfield, B.E., Dionne, M.S., Schneider, D.S., and Freitag, N.E. (2003). Exploration of host–pathogen interactions using Listeria monocytogenes and Drosophila melanogaster. Cellular microbiology *5*, 901-911.

200. Joyce, E.A., Kawale, A., Censini, S., Kim, C.C., Covacci, A., and Falkow, S. (2004). LuxS is required for persistent pneumococcal carriage and expression of virulence and biosynthesis genes. Infection and immunity *72*, 2964-2975.

201. Kain, J., Stokes, C., Gaudry, Q., Song, X., Foley, J., Wilson, R., and de Bivort, B. (2013). Leg-tracking and automated behavioural classification in Drosophila. Nat Commun *4*, 1910.

202. Mendes, C.S., Bartos, I., Akay, T., Márka, S., and Mann, R.S. (2013). Quantification of gait parameters in freely walking wild type and sensory deprived Drosophila melanogaster. eLife *2*.

203. Elmquist, J.K. (1997). Mechanisms of CNS response to systemic immune challenge: the febrile response. Trends in neurosciences (Regular ed.) *20*, 565-570.

204. Pham, L.N., Dionne, M.S., Shirasu-Hiza, M., and Schneider, D.S. (2007). A Specific Primed Immune Response in <italic>Drosophila</italic> Is Dependent on Phagocytes. PLoS Pathog *3*, e26.

205. Calder, P.C. (2006). Branched-chain amino acids and immunity. The Journal of nutrition *136*, 288S-293S.

206. Wong, A., Chaston JM, Douglas AE (2013). The inconstant gut microbiota of Drosophila species revealed by 16S rRNA gene analysis. The ISME Journal *7*, 1922-1932.

207. Erkosar, B. (2014). Transient adult microbiota, gut homeostasis and longevity: novel insights from the Drosophila model. FEBS letters *588*, 4250-4257.

208. Deretic, V. (2013). Autophagy in infection, inflammation and immunity. Nature reviews. Immunology *13*, 722-737.

209. Berchtold, D., and Walther, T.C. (2009). TORC2 Plasma Membrane Localization Is Essential for Cell Viability and Restricted to a Distinct Domain. Molecular Biology of the Cell *20*, 1565-1575.

210. Rispal D, E.S., Stahl M, Vaga S, Bodenmiller B, Abraham Y, Filipuzzi I, Movva NR, Aebersold R, Helliwell SB, Loewith R (2015). Target of Rapamycin Complex 2 Regulates Actin Polarization and Endocytosis via Multiple Pathways. The Journal of biological chemistry *290*, 14963-14978.

211. Alberts B, J.A., Lewis J, et al. (2002). Molecular Biology of the Cell. 4th edition, (New York: Garland Science).

212. Shirasu-Hiza, M.M.S., David S. (2007). Confronting physiology: how do infected flies die? Cellular microbiology *9*, 2775-2783.

213. Miron, M. (2001). Regulation of translation via TOR signaling: insights from Drosophila melanogaster. The Journal of nutrition *131*, 2988S-2993S.

214. Peng, J.Z.P., Kubli E (2005). Drosophila sex-peptide stimulates female innate immune system after mating via the Toll and Imd pathways. Current biology *15*, 1690-1694.

215. McKean, K.A.N., L. (2001). Increased sexual activity reduces male immune function in Drosophila melanogaster. Proceedings of the National Academy of Sciences - PNAS *98*, 7904-7909.

216. Stroschein-Stevenson SL, F.E., O'Farrell PH, Johnson AD (2006). Identification of Drosophila gene products required for phagocytosis of Candida albicans. PLoS biology *4*, e4.

217. Peltan A, B.L., Matthews G, Sweeney ST, Smith DF (2012). Identification of Drosophila gene products required for phagocytosis of Leishmania donovani. PloS one *7*, e51831.

218. Landis G, S.J., Tower J (2012). Gene expression changes in response to aging compared to heat stress, oxidative stress and ionizing radiation in Drosophila melanogaster. Aging (Albany, NY.) *4*, 768-789.

219. Carlson, K.A.G.K.P.A.C.D.Y.F.E.J.Z.C.H.L. (2015). Genome-Wide Gene Expression in relation to Age in Large Laboratory Cohorts of Drosophila melanogaster. Genetics research international *2015*, 1-19.

220. McDonald, M.J.R., M. (2001). Microarray analysis and organization of circadian gene expression in Drosophila. Cell (Cambridge) *107*, 567-578.

221. Wijnen H, N.F., Boothroyd C, Claridge-Chang A, Young MW (2006). Control of daily transcript oscillations in Drosophila by light and the circadian clock. PLoS genetics *2*, e39.

222. Keegan, K., Pradhan S, Wang JP, Allada R (2007). Meta-analysis of Drosophila circadian microarray studies identifies a novel set of rhythmically expressed genes. PLoS computational biology *3*, e208.

223. Barzilai, N. (2012). The critical role of metabolic pathways in aging. Diabetes (New York, N.Y.) *61*, 1315-1322.

224. Cao, R.L.A.C.H.L.B.O.K. (2010). Mammalian target of rapamycin signaling modulates photic entrainment of the suprachiasmatic circadian clock. The Journal of neuroscience *30*, 6302-6314.

Appendix 1: Relates to Chapter II

Figure S1

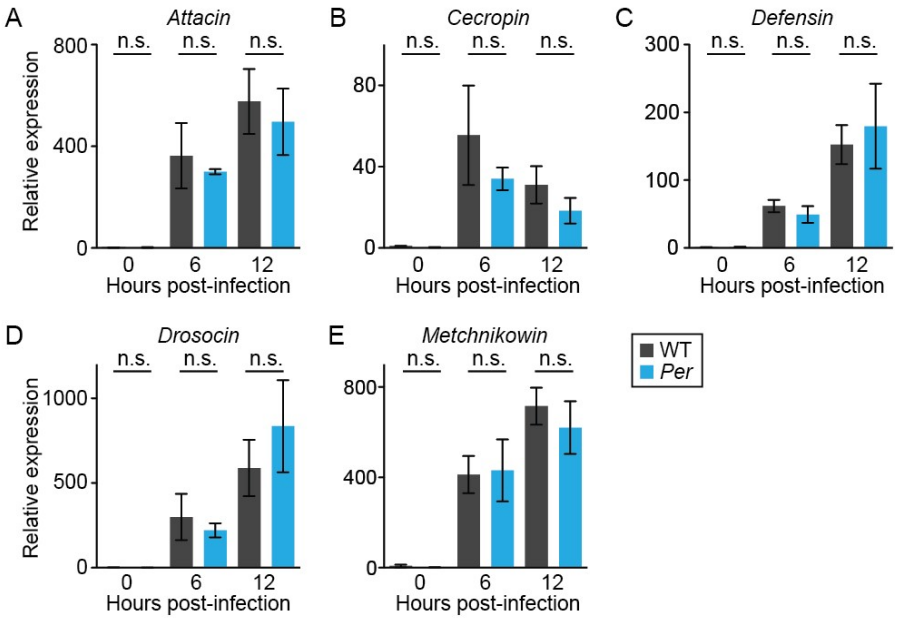

Figure S1: *Per*^{01} mutants do not have increased AMP induction following infection with *B. cepacia*. Consistent with a tolerance phenotype, antimicrobial peptide (AMP) induction did not differ between *Per*^{01} mutants and wild-type flies after *B. cepacia* infection as shown by qRT-PCR of: A) *Attacin* B) *Cecropin* C) *Defensin* D) *Drosocin* and E) *Metchnikowin*. (3 samples of n=6 flies, n.s. for all time points). Error bars represent the mean ± S.E.M.; n.s.=not significant ($p>0.05$). Relates to Figure 1.

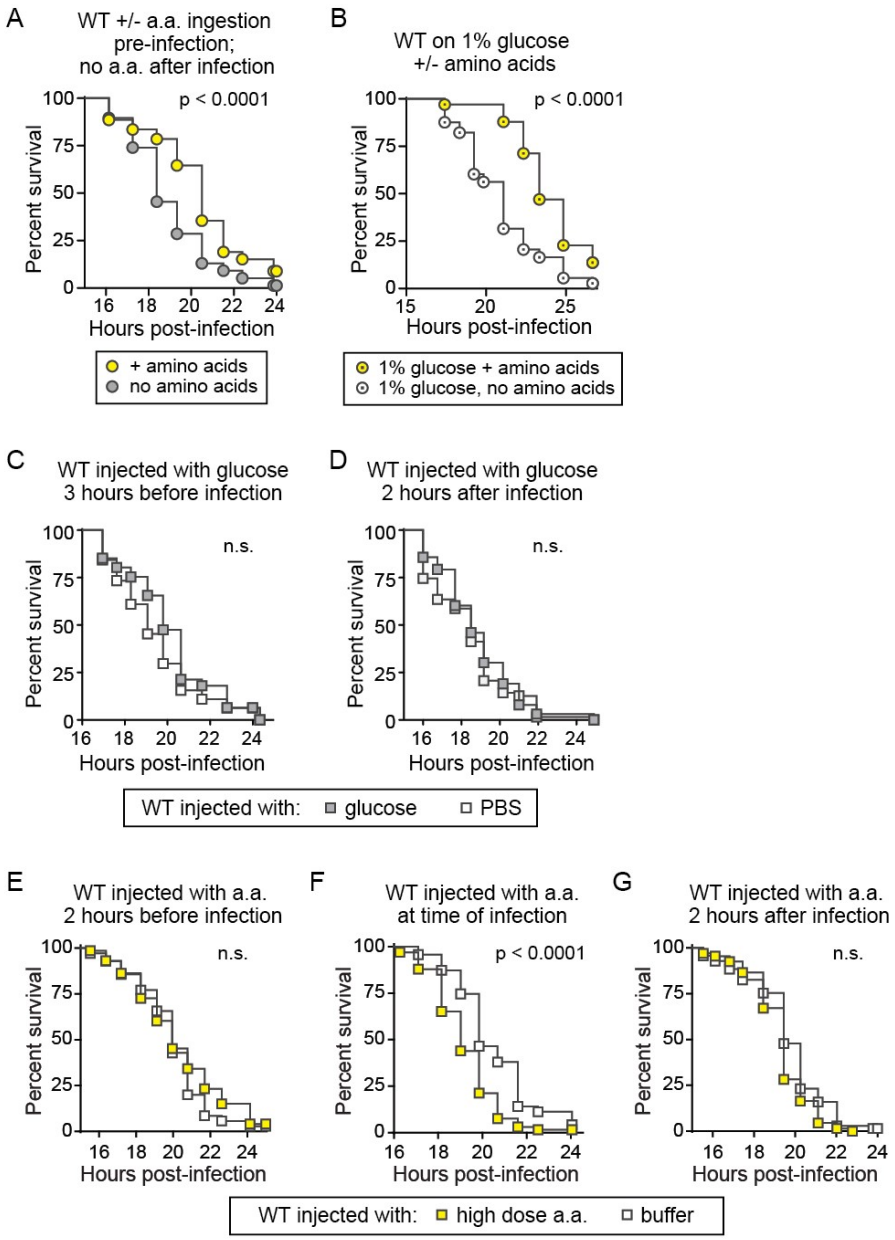

Figure S2: Wild-type flies fed or injected with certain nutrients at specific times before or after infection with *B. cepecia* exhibit altered survival kinetics. A) Dietary amino acids before infection can still confer a survival benefit when they are unavailable post-infection. Flies fed 5% glucose with or without amino acids were all switched to 5% glucose without amino acids upon infection. Flies that received the amino acid-supplemented diet prior to infection (n=79) survived infection longer than flies that did not receive dietary amino acids prior to infection (n=77, p<0.0001). B) Food containing 1% glucose plus amino acids improves survival of infection. Supplementing 1% glucose with amino acids

increased survival time relative to the 1% glucose-only diet (glucose alone, n=73; with a.a., n=66, $p<0.0001$). Glucose does not increase survival when injected into flies C) >2 hours before (buffer n=64, glucose n=61, n.s.) or D) ≥2 hours after infection with *B. cepacia* (buffer n=66, glucose n=67, n.s.). Amino acid (a.a) injection does not increase survival when injected E) before (buffer n=70, amino acids n=73, n.s.), F) during (buffer n=71, amino acids n=66, $p<0.0001$), or G) after infection with *B. cepacia* (buffer n=69, amino acids n=67, n.s.). n.s.=not significant ($p>0.05$). Relates to Figure 4.

Figure S3

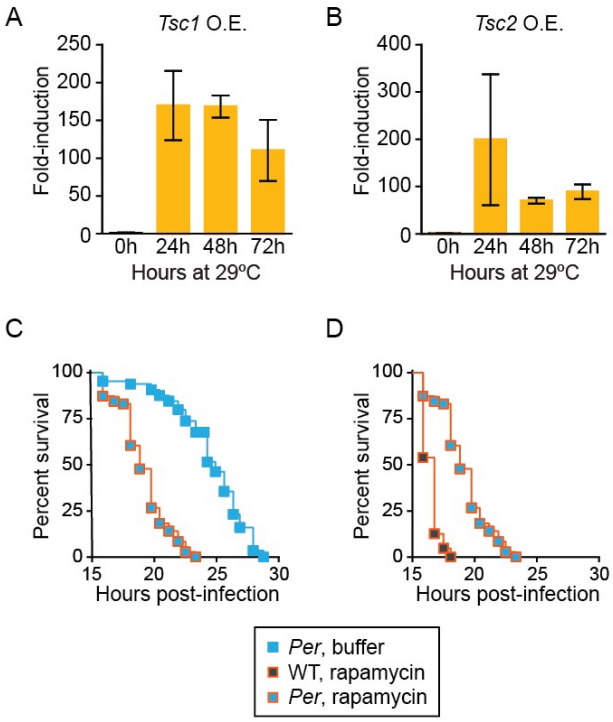

Figure S3: TORC1 characterization. We performed qRT-PCR confirmation of *Tsc1/2* over-expression (O.E.) after heat shock. Shown here are qRT-PCR analyses of transgene expression for A) *Tsc1* and B) *Tsc2* after 0, 24, 48, and 72 hours at 29°C in flies co-expressing *tubulin-Gal80ts* and *tubulin-Gal4* with *UAS>Tsc1* and *UAS>Tsc2*. (3 samples of n=6 flies for each time point, $p<0.05$ for 0 hr vs. all time points except *Tsc1* 72 hr and *Tsc2* 24 hr). Reducing TORC1 activity does not eliminate the survival advantage of Per^{01} mutants. C) Rapamycin injection decreases survival of Per^{01} mutants relative to buffer alone (n=71, n=65; $p<0.0001$), suggesting that Per is upstream, rather than downstream, of TORC1 in this context [224]. D) When injected with rapamycin, Per^{01} mutants survive longer than wild type (n=71, n=63; $p<0.0001$). Error bars represent the mean ± S.E.M. Relates to Figure 5.

Figure S4

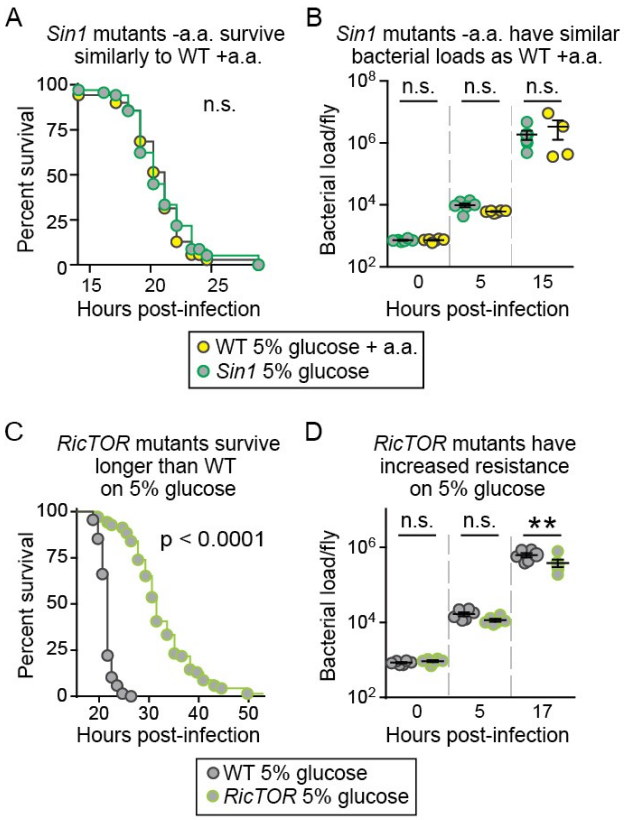

Figure S4: Inhibition of TORC2 leads to changes in both tolerance and resistance of infection. A) *Sin1*^{e03756} mutants deprived of amino acids (n=69) do not survive longer than wild-type flies fed amino acids (n=70, n.s.) and B) have similar bacterial loads following infection with *B. cepacia* (n≥4, n.s. all time points). C) *rictor*$^{\Delta 2}$ mutants (n=69) have a survival advantage over wild-type flies (n=68) when fed 5% glucose (p<0.0001) and D) have increased resistance to infection with *B. cepacia* (n≥5 all time points, 0 hrs n.s., 5 hrs n.s., 17 hrs p<0.0022). n.s.=not significant (p>0.05); **=p≤0.01. Error bars represent the mean ± S.E.M. Relates to Figure 6.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Fly cultures and media

Flies were bred and raised on standard food containing cornmeal, molasses, and yeast, with a total concentration of 5-10% sugar and 2-3% yeast. The mixture was boiled for 25 minutes, then let cool for 45 minutes, or until the temperature reached 65°C or less. Methylparaben (Tegosept, 50 g in 300 mL ethanol) and 180 mL of propionic acid were then added as antimicrobial agents. Sugar-only foods contained 1% (w/v) agar (Fisher #A360-500 or Alfa Aesar #A10752) and different concentrations (w/v) of glucose (Fisher #BP350-1 or #BP350-500) dissolved in ddH_20. Amino acid-containing food contained 1% agar, 5% glucose, and 2% (w/v) amino acid mixture containing all 20 amino acids (Sunrise Science #1360-030). For experiments involving diets other than standard food, flies were switched to the experimental food ~24 hours before infection and remained on it thereafter, except for S2A. The AMP, melanization, phagocytosis, and rapamycin survival assays were performed with flies on standard food. Mutant assays were performed with flies on 5% glucose + amino acids.

Bacterial cultures

B. cepacia was grown overnight at 29°C in 5 mL standing Brain Heart Infusion (Teknova), resuspended in sterile PBS (Invitrogen #003000), and diluted to an OD_{600} of 0.0001 for injection into flies (long infection at 18°C) or OD_{600} of 0.025 or 0.0275 (short infection at 29°C). Except for Figure 1A & C, all subsequent experiments utilized the short infection protocol, which typically caused death within 24 hours of infection.

Injections

All experiments, including injections, were performed with age-matched flies. All flies except *tub>Gal80-ts; tub>Gal4; UAS-Tsc1/Tsc2* flies were raised at 25°C, 55–65% humidity on standard food and entrained to a 12h:12h light:dark cycle in a Darwin Chambers incubator for at least 3 days prior to infection. For injections, flies were lightly anesthetized with CO_2. Injections were carried out with a 10 µL Drummond Scientific glass capillary needle (#3-000-210-G), machine-pulled by a Sutter Instrument Co. machine (Model P-30). A custom-modified Tritech microinjector was used to inject 50 nL of liquid into each fly. Volume was calibrated by measuring the diameter of an expelled drop in halocarbon oil 700 (Sigma

#H8898) under a layer of mineral oil (Sigma #M8410). All injections were performed between (Zeitgeber time) ZT7.5 and ZT10.5 to minimize variability from circadian effects on immunity. After injection with *B. cepacia*, flies were incubated at 29°C, except for long infections and melanization assays, in which flies were incubated at 18°C. When examining the time window for efficacy of dietary nutrients, we performed two injections, one with glucose or amino acids and one with *B. cepacia*, both 50 nL, except when nutrients were administered at the time of infection, as a co-injection, mixed with bacteria. For co-injection of glucose, *B. cepacia* was resuspended in either PBS or a solution of 5% glucose dissolved in PBS. For co-injection of amino acids, *B. cepacia* was resuspended in either PBS or a solution of amino acids dissolved in PBS (low dose 11 µg/mL or high dose 220 µg/ml). For co-injection of rapamycin, OR flies were injected with *B. cepacia* suspended in either buffer alone or in buffer containing 0.2 mg/mL rapamycin (Calbiochem #553210).

Survival assays

Except where otherwise noted, approximately 60 male flies per genotype per condition were assayed for each survival curve and divided into vials with approximately 20 flies per vial. In each experiment, approximately 20 flies of each genotype and each condition were also injected with media to control for the effects of wounding alone. Data were converted to Kaplan-Meier format using custom Excel-based software called Count the Dead (J. Shirasu-Hiza).

Bacterial load quantitation

Following challenge with microbes, approximately six individual flies were collected at each time point. Time points analyzed typically included just after infection (0h); mid-infection (6h); and late in infection (12-16h) just before the lethal phase of infection, when bacterial load measurements become highly variable. Individual flies were homogenized in 100 µL PBS, diluted serially and plated on standard LB agar plates. Colonies were counted after growth overnight at room temperature or 29°C. To ensure lack of pre-existing infection or media contamination, three PBS-injected flies for each genotype and condition were tested for bacterial load. Typically no colonies were observed for uninfected flies.

Quantitative real-time RT-PCR

RNA was extracted from three or four samples of 5-6 adult flies using TRIZOL (Invitrogen) according to the manufacturer's directions. RNA was treated with DNAse I (Invitrogen #18068-015), followed by reverse transcription using the First Strand cDNA Synthesis Kit (Thermo Scientific #K1622), priming with random hexamers. Quantitative PCR was performed in 25 µL reactions with Roche Fast Start Universal SYBR Green Master Mix (#04913850001) on a Stratagene Mx3000P or a Bio-Rad CFX Connect Real-Time System. The cycling conditions used were: Hold 95°C for 10 minutes, then 40 cycles of 95°C for 15s, 58°C for 30s, 72°C for 30s, then a final sequence of 95°C for 1 minute, 57°C for 30s, and 95°C for 30s. All calculated gene expression values were initially normalized to the transcript level of ribosomal protein 4, *Rpl1*, prior to further analysis. Primer sequences are listed here:

Oligonucleotide sequences.

Gene	**Left primer**	**Right primer**
AttA	CACAATGTGGTGGGTCAGG	GGCACCATGACCAGCATT
CecA1	TCTTCGTTTTCGTCGCTCTC	CTTGTTGAGCGATTCCCAGT
Def	TTCTCGTGGCTATCGCTTTT	GGAGAGTAGGTCGCATGTGG
Dipt	ACCGCAGTACCCACTCAATC	CCCAAGTGCTGTCCATATCC
Dro	CCATCGAGGATCACCTGACT	CTTTAGGCGGGCAGAATG
Drs	GTACTTGTTCGCCCTCTTCG	CTTGCACACACGACGACAG
Mtk	TCTTGGAGCGATTTTTCTGG	TCTGCCAGCACTGATGTAGC
Rpl1	TCCACCTTGAAGAAGGGCTA	TTGCGGATCTCCTCAGACTT
Tsc1	GTAAACACACCTTGTCCAAGCAGC	TGACAGATGGATAGACGGAACCAC
Tsc2	ACACATAGACAACAACGAGAGG	AAGAGATATCATGGCAGGATGC

Melanization assays

Wound site and systemic melanization were detected by visual inspection. Two groups of ~20 flies were injected with *B. cepacia* (OD_{600} 0.005 – 0.05) and incubated at 18°C. One day after infection, flies from

one group were each visually inspected for wound site melanization, a small deposit of melanin at the site of injection. Three to five days after infection, flies from the second group were visually inspected for the systemic melanization response, characterized by one or more melanin deposits located anywhere on the body other than at the injection site.

Bead inhibition of phagocytosis

Phagocytosis was inhibited by injection of fluorescent 1.0 µm polystyrene beads (Molecular Probes #F13080). 200 µl of beads in solution were washed three times in sterile PBS and resuspended in PBS for a final volume of 30 µl. 7-10 day old male flies were injected with either 100 nL of bead solution or buffer (PBS) as a control. After 2 days, flies were injected with *B. cepecia* at an OD_{600} of 0.025 (see Survival Assays, above). p-values for survival curves were obtained by log-rank analysis. To confirm inhibition of phagocytosis, an *in vivo* phagocytosis assay was performed 2-3 days post bead injection on a small subset of bead- and PBS-injected flies. Briefly, flies were injected with 50 nL of 20 mg/mL pHrodo-labeled *S. aureus* in PBS (Molecular Probes #A10010) and incubated for 20-30 minutes. The dorsal surfaces of the flies were superglued to coverslips and imaged using epifluorescence illumination with a Nikon upright fluorescent microscope with HQ CCD using Nikon Elements software. Phagocytosis was completely inhibited within 2-3 days of bead injection.

Starvation assay

Using the DAM5 system (TriKinetics), age-matched 5-7 day-old male flies were incubated at 25ºC, 55-65% humidity in a 12h:12h light:dark cycle on media containing 1% agar alone. For each genotype, n=16 flies per experiment. Flies were counted as dead following an absence of beam crossings for the remainder of the experiment. Similar results were obtained when starvation experiments were performed using visual inspection to confirm death.

Metabolic storage assays

For metabolic assays, samples were prepared by homogenizing 8 adult male flies (5-10 days old) in 200 ul Tris-EDTA (TE; 10 mM Tris, 1 mM EDTA, pH 8) + 0.1% Triton X-100, then freezing at -20ºC.

Triglyceride levels were normalized to baseline levels of glycerol in each sample; glycerol levels did not vary significantly between conditions. Briefly, the glucose and trehalose levels were measured using a glucose oxidase reagent (Pointe Scientific) dissolved in ddH_2O. Glycogen was measured using the same reagent supplemented with 1 U/mL amyloglucosidase (Sigma #10115-1G-F). This reaction was blanked against the glucose reaction for each given sample. All reactions were performed on 96-well tissue culture plates with 0.2 mL reaction mixture + 0.02 mL sample. Plates were incubated at 37ºC for one hour, and then absorbance for each well was measured at 490 nm using a BioRad Model 680 Microplate Reader. The values obtained for each metabolic reserve were normalized to the average weight of flies of that given genotype. Average weight was determined by measuring the mass of 8 samples of 50 adult male flies from each genotype. The resulting ratio of wild type to *Per*^{01} mass was 1.054; in no case did this adjustment convert a result from being insignificant to significant, or vice versa. These values were then normalized to the mean obtained from the wild type values and plotted with the normalized SEM.

Feeding assays

For the radiolabeled feeding assay, flies were maintained on medium supplemented with [α-^{32}P]-dCTP for 24 hours and then collected for liquid scintillation to measure accumulated ^{32}P. Aliquots of radiolabeled food were used to convert scintillation counts to volume. For the CAFE assay, flies were fed liquid food solutions of yeast extract and sucrose in glass microcapillaries that facilitated the direct measurement of volume consumed.

Protein extraction and Western blotting

Ten whole flies were homogenized in 250 µL of extraction buffer: 1X PBS, 0.1% Triton X-100, complete protease inhibitor cocktail (Roche), 1X LDS sample loading buffer (Life Technologies), 40 mM DTT. Briefly, samples were resolved on a 4-15% SDS polyacrylamide tris-glycine gel, transferred for 1 hour at 4°C at 100 volts, and immunoblotted using standard procedures. Membranes were blocked for 1 hour using 3% BSA in TBS-T (1XTBS + 0.1% Tween 20). Antibodies were diluted in 3% BSA in TBS-T, at 1:1000 for anti-phospho-S6K (Thr398) (Cell Signaling #9209) and 1:10,000 for anti-actin-HRP (Sigma #A3854**),** and applied to membrane overnight while shaking at 4°C. Blots were washed three times in

PBS-T for 10 minutes at room temperature. Secondary antibody anti-rabbit HRP (Cell Signaling #7074) was applied to membranes at a concentration of 1:2000 in 3% BSA in TBS-T for 2 hours shaking at room temperature, followed by three washes in PBS-T for 10 minutes at room temperature. Blots were developed using chemiluminescence substrate (Millipore #WBKLS0500). Images were captured using an Image Station CCD camera (Kodak). Densitometry was performed using Image J gel analysis package. Quantification of phospho-S6K signal was performed by measuring the mean intensities of the phospho-S6K bands and normalizing to the mean intensities of actin controls for each sample.

Appendix 2: Chapter IV Detailed Experimental Methods

Background subtraction

In order to distinguish pixels containing the fly from pixels not containing the fly, we use an image of the empty apparatus as the background reference image (BRI). By comparing the grayscale values in each pixel of the first video frame against the equally-positioned pixel in the BRI, we can then identify the pixels which contain the fly's image. If the pixel on the template frame is darker than that of the comparison frame, it means that the pixel on the template frame belongs to the fly.

We use a threshold constant C_0 to correct for pixel grayscale value differences that likely come from fluctuations in the recording instrument. This ensures that the difference between the template frame and the comparison frame is due to noise, and not the actual appearance of the fly.

Specifically, for a pixel (x, y), if the difference in grayscale value between comparison and template frames is greater than threshold C_0, the following relationship holds:

$$I_{template(x,y)} - I_{comparison(x,y)} < -C_0$$

For grayscale value of this pixel we set:

$$I_{template(x,y)} = I_{comparison(x,y)}$$

Thus if grayscale value of a pixel (x,y) is C_0 less than that of the background frame, it is selected as a fly pixel and recorded in a "fly" matrix as fly(x,y) = 1, otherwise fly(x,y) = 0. Some noise still remains after applying this threshold, but the noise pixels are generally not grouped together in large clusters. Rather, noise pixels are found scattered throughout the matrix. Although noise pixels can merge as small objects, these are much smaller than the fly. We use a threshold area to exclude and erase small objects, leaving only the image of the fly. Thus, we have constructed a binary image, "fly".

The extent of a fly's movement from one frame to the next can be observed as those pixels with different grayscale values from the previous "comparison" frame. However, if the fly in the relevant and comparison frames occupies the same area, the overlap area cannot be erased. In order to solve this

problem we use multiple comparison frames to avoid overlapping. For every 10,000 frames (1000 second), we uniformly pick seven frames to compare with the template, and then the template frame becomes the background frame to compare with the 10000 frames.

In comparing frames, we must again correct for noise from the recording instruments. To this end, we analyze video of a dead fly in the apparatus. In the absence of random fluctuations in intensity, each frame in the video should be the same. Thirty pairs of frames are randomly chosen from the video and the difference in each pixel's grayscale value is calculated and recorded in a matrix. The non-zero elements in this matrix reflect fluctuations from the recording system, and the value of elements indicates the intensity of the fluctuation.

In our experiments, the frame size was 1280×960, and the grayscale value ranged from 0 (black) to 255 (white). We set threshold C1=15, which allowed us to remove 99.99% of noise.

Center displacement calculation

In order to distinguish between locomotion and grooming, we need to know whether the fly's center is moving. Therefore, we calculate the position of the fly's center by averaging the positions of all of the fly's pixels.

Suppose (x_1,y_1), (x_2,y_2), … (x_n,y_n) are all the points satisfied by the fly(x_i,y_i) = 1. The fly's position can be calculated by:

$$(x, y) = \frac{\sum_{i=1}^{n}(x_i, y_i)}{\mathrm{n}}$$

Then locomotion can be quantified as the difference in position of the fly's center from the previous frame. Since the tube is approximately one dimensional, when calculating locomotion we ignore movement perpendicular to the tube.

Core and periphery selection

Flies groom several parts of their bodies, including legs, head, wing, and abdomen. However, in general, all types of grooming are characterized by a relatively stationary fly core surrounded by a moving

periphery. Therefore, it's necessary to separate the fly in to core and periphery to calculate their movements individually.

Since a fly's periphery areas are lighter than its core area, we can use an intermediate value function to separate the range of grayscale values into core and periphery. The intermediate value is calculated for each individual fly depending on its grayscale distribution. Pixels darker than average grayscale values are selected as core pixels. Similarly, pixels lighter than average grayscale values are selected as core pixels. When selecting the core and periphery we assume the core and periphery are approximately equal in size. Before the fly is separated into the core and periphery, we first calculate the average grayscale value μ and standard deviation $б$ for each fly as shown:

Suppose there are n pixels (x_1,y_1), (x_2,y_2), … (x_n, y_n) satisfied by $fly(x_i,y_i) = 1$. The average grayscale value of the fly can be calculated by:

$$\mu = \frac{\sum_{i=1}^{n} I_{frame(x_i,y_i)}}{n}$$

And standard deviation:

$$б = \frac{\sum_{i=1}^{n} (I_{frame(x_i,y_i)} - \mu)^2}{n}$$

To select core pixels:

Pixels (x,y) that satisfy the condition below will be selected as core pixels, recorded as core(x, y) = 1, otherwise core(x,y) = 0.

$$\frac{I_{frame(x,y)} - \mu}{б} > C_2$$

To select periphery pixels:

Pixels (x,y) that satisfy the below condition will be selected as periphery pixels, recorded as periphery(x,y) = 1, otherwise periphery(x,y) = 0.

$$\frac{I_{frame(x,y)} - \mu}{б} < C_2$$

C_2=0.7 making the number of pixels in core and periphery approximately the same.

Next, to calculate the movement of the fly's core (CC), we quantify the number of core pixels that are different from the previous frame, or in other words, the number of core pixels that do not overlap in the two neighboring frames.

$$core(x,y,t) \neq core(x,y,t-1)$$

The pixels not in the overlap area are recorded as difcore(x ,y, t) = 1, and the total number of pixels that show core movement is calculated:

$$CC(t) = sum(difcore(x,y,t))$$

The number of periphery pixels that are different from previous frame (CP) is calculated similarly.

Quantifying the movement of the fly's core and periphery does not yet allow us to determine whether the fly is grooming based on low core movement coupled with high periphery movement. For example, a grooming event might register as a false negative if the fly's body slides during grooming. Alternatively, a small grooming movement might register as a false negative. To avoid these errors, we focus only on grooming behaviors lasting for more than three seconds.

Entrainment of the classifier

We use the following KNN method to distinguish target frames that may show grooming behavior:

The first step in the process is to train the classifier. We manually pick out N frames as training samples and label them with their specific class of behaviors (i.e. grooming, moving and resting). Then we can map these frames into DS-CP-CC space with the coordinate (ds_i, cp_i, cc_i) $(i \in \{1: N\})$, where each point in the space corresponds to one frame from the video. We save the three parameters of

training samples in to an N by 3 matrix, and their labels into an N by 1 matrix, with 1 for grooming, 2 for moving and 3 for resting.

Next we test the KNN classifier with another M frames (testing samples), which are manually labeled and not used as training samples. We map each testing frame to a point (ds_j, cp_j, cc_j) ($j \in \{1:M\}$) in the feature space, and compute the Euclidean distances from the testing point to each training sample. Then this testing point is classified by assigning the label which is most frequent among the k training samples nearest to it. After labelling all testing samples by classifier, we compare the result with manual labels, and adjust the k value, to validate and optimize our classifier. To balance accuracy and efficiency, we set k=10 in our algorithm.